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# Analysis of Mutations in Superoxide Dismutase-1 and the Protective Effect of Heat Shock Proteins against mutant-SOD1 Toxicity

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*To all my family and friends*

*"My time will come".....Mendel*

## Abstract

Genetic studies have revealed over 100 mutations in the gene encoding superoxide dismutase type-1 (SOD1) that cause Familial Amyotrophic Lateral Sclerosis (FALS). For the purpose of this thesis an *in vitro* model has been developed by stably over-expressing wild type (wt), G93A or G93R mutant SOD1 in neuronally derived ND7 cell line. It was found that wt-SOD1 could provide protection against a range of cell stresses including serum removal (plus retinoic acid); IFN-γ, staurosporine; camptothecin; ischemia/reoxygenation; glutamate; and hydrogen peroxide ($H_2O_2$). In contrast, both mutant forms of SOD1 enhanced cell death with the G93R mutation being the more severe of the two mutations tested. Hence, the disease-associated mutations convert wt-SOD1 from a protective anti-apoptotic protein to a pro-apoptotic form. Most of the above stresses are FALS relevant stresses, which primarily induce apoptotic cell death in ND7 cells, implicated in FALS pathology.

The neuroprotective effect of various heat shock proteins (Hsps) in the above system was studied, utilising a Herpes Simplex Virus (HSV) - based gene delivery system. For the first time, it was demonstrated that in an *in vitro* model of mutant-SOD1-induced toxicity, the exogenous expression of Hsp27 and/or Hsp70 protects both G93A and G93R-SOD1-mutant expressing cells, under all the stresses tested, and the dual expression of Hsp27 and Hsp70 is more effective in protecting against mutant-SOD1 cytotoxicity than either Hsp individually; as assessed by trypan blue and TUNEL analysis. In addition, G93A and G93R-SOD1 mutant expressing cells were markedly protected by caspase-8 and caspase-9 inhibition. However no additive protective effect of Hsp and caspase inhibitor was observed.

To further investigate the protective effect of wt-SOD1 and damaging effect of the mutant, an HSV-based gene delivery system was utilised. Primary cultures of dorsal root ganglia (DRGs) from postnatal rats, wild-type mice, transgenic Hsp27 and transgenic Hsp70 mice were infected with SOD1 viruses for wt-SOD1 and G93R-mutant SOD1 and subjected to stresses of NGF withdrawal, IFN-γ and staurosporine treatment. Finally, the DRG experiments were repeated with additional delivery of Hsps via HSV vectors to further

investigate the protective effect of the Hsps. These experiments provided further evidence on the protective role of Hsp27 and/or Hsp70 against mutant-SOD1 induced toxicity.

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And finally I would like to thank my family and friends for being there for me.

## Declaration

I declare all the results incorporated within this thesis constitute my own work and findings. The SOD1 stable cell lines and SOD1 viruses were constructed in our laboratory by Dr Yolanda Collaco-Moraes. The Hsp viruses were constructed in our laboratory by Dr Marcus Wagstaff. The transgenic Hsp27 (Tg-Hsp27) mice were a gift from Professor J. de Belleroche, Imperial College, London, UK. The transgenic Hsp70 (Tg-Hsp70) mice were a gift from Dr. C.E. Angelidis, Dalhousie University, Canada. The ApoE isoforms were a gift from Dr B.S. Thilakawardhana, Royal Free Hospital, University College London, UK.

## Publications

[1] Patel Y., Collaco Moraes Y., Latchman D., Coffin R., de Belleroche J. *Neuroprotective effects of copper/zinc-dependent superoxide dismutase against a wide variety of death-inducing stimuli and proapoptotic effect of familial amyotrophic lateral sclerosis mutations.* Mol. Brain Res. 2002; 109(1-2):189-97

[2] Patel Y., Payne-Smith M., de Belleroche J., Latchman D. *Hsp27 and Hsp70 administered in combination have a potent protective effect against FALS-associated SOD1-mutant induced cell death in mammalian neuronal cells.* Mol. Brain Res. 2005; 134: 256-274

[3] Patel Y., Payne-Smith M., de Belleroche J., Latchman D. *Hsp27 and Hsp70 administered in combination have a potent protective effect against FALS-associated SOD1-mutant induced cell death in a primary neuronal cell culture system (in preparation)*

[4] Patel Y., Shah K, Thilakawardhana BS, Payne-Smith M., de Belleroche J., Latchman D. *The differing effects of ApoE isoforms on FALS-associated SOD1-mutant induced cell death in mammalian neuronal cells (in preparation)*

## Abbreviations

| | |
|---|---|
| Aβ | β-amyloid peptide |
| AD | Alzheimer's Disease |
| AIF | apoptosis inducing factor |
| Akt | protein kinase B |
| ALS | amyotrophic lateral sclerosis |
| ANOVA | analysis of variance |
| Apaf-1 | apoptotic protease-activating factor 1 |
| AR | androgen receptor |
| AR-PD | autosomal recessive PD |
| ATP/ADP/AMP | adenosine trisphosphate/disphosphate/monophosphate |
| BACE | β-amyloid cleaving enzyme |
| βAPP | β-amyloid precursor protein |
| bp | base pairs |
| BSA | bovine serum albumine |
| CCCP | carbonyl cyanide chlorophenylhydrazone |
| cDNA | complementary DNA |
| CFTR | cystic fibrosis transmembrane conductance regulator |
| CHIP | carboxyl terminus of the Hsc70 -interacting protein |
| CIAP | calf intestine alkaline phosphatase |
| CMV | cytomegalovirus |
| CMV-IE | cytomegalovirus immediate-early |
| DA | dopamine, 3, 4-dihydroxyphenethylamine |
| $ddH_2O$ | double distilled water |
| DIABLO | direct IAP binding protein |
| DMEM | Dulbecco's modified Eagle's medium |
| DMSO | dimethylsulphoxide |
| DNA | deoxyribonucleic acid |
| dNTPs | deoxynucleotide triphosphates |
| DRG | Dorsal root ganglia |
| DRPLA | dentatorubropallidoluysian atrophy |

| | |
|---|---|
| E | early gene |
| *E.coli* | *Escherichia coli* |
| ECL | enhanced chemiluminescence |
| EDTA | diaminoethanetetra-acetic acid, disodium salt |
| ER | endoplasmic reticulum |
| FADD | Fas-associated protein with death domain |
| FALS | Familial Amyotrophic Lateral Sclerosis |
| FCS | fetal calf serum |
| FGM | full growth medium |
| FKBP | FK506 binding protein |
| GAD | glutamic acid decarboxylase |
| gad | gracile axonal dystrophy |
| GDNF | glial derived neurotrophic factor |
| GFP | green fluorescent protein |
| GR | glucocorticoid hormone receptor |
| HBSS | Hank's balanced salt solution |
| HD | Huntington's Disease |
| HMBA | hexamethylene bisacetamide |
| HNE | 4-hydroxy-2-trans-nonenal |
| HO1 | haem oxygenase 1 |
| HSE | heat shock element |
| HSF | heat shock factor |
| Hsp | heat shock protein |
| HSV-1 | herpes simplex virus type 1 |
| ICP | infected cell protein |
| IE | immediate-early gene |
| $InsP_3$ | inositol 1,4,5-trisphosphate |
| Kb | kilobase |
| kDa | kiloDalton |
| L | late gene |
| L-15 | medium Leibovitz's L-15 medium |
| LAT | latency associated transcript |

| | |
|---|---|
| LAT P | latency associated transcript promoter |
| LB | Lewy body |
| LTR | long terminal repeat |
| m.o.i | multiplicity of infection |
| MAP | mitogen-activated protein |
| MAPK | mitogen activated protein kinase |
| MCS | multiple cloning site |
| MFI | mean fluorescent intensity |
| MJD | Machado–Joseph disease |
| MMTV | murine mammary tumor virus |
| MnSOD | Manganese SOD (SOD2) |
| $MPP^+$ | methyl-4-phenylpyridinium ion |
| MPTP | 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine |
| mRNA | messenger RNA |
| NFT | Neurofibrillary tangles |
| NGF | nerve growth factor |
| NMDA | N-methyl-D-aspartate |
| Pael-R | parkin-associated endothelin receptor-like receptor |
| PAGE | polyacrylamide gel electrophoresis |
| Paraquat | 1,1'-dimethyl-4,4'-5 bipyridinium |
| PBS | phosphate buffered saline |
| PCD | Programmed Cell Death |
| PD | Parkinson's Disease |
| PDGF | platelet-derived growth factor |
| pfu | plaque forming units |
| PINK1 | PTEN-induced kinase 1 |
| PKC | protein kinase C |
| polyQ | polyglutamine |
| PS | phosphatidylserine |
| RA | retinoic acid |
| RNA | ribonucleic acid |
| ROS | reactive oxygen species |

| | |
|---|---|
| rpm | revolutions per minute |
| RSV | Rous Sarcoma Virus |
| SALS | sporadic Amyotrophic Lateral Sclerosis |
| SBMA | spinal and bulbar muscular atrophy |
| SCA1 | spinocerebellar ataxia type 1 |
| SD | standard deviation |
| SDS | sodium dodecyl sulphate |
| SFM | serum free medium |
| SMAC | second mitochondrion derived activator of caspases |
| SOD-1 | superoxide dismutase type-1 |
| SUMO | small ubiquitin-related modifier |
| TAE | tris-acetate-EDTA-buffer |
| TdT | terminal deoxynucleotidyl transferase |
| TEMED | N,N,N',N'-tetramethyl-ethylenediamine |
| TPR | tetratricopeptide repeat |
| TUNEL | TdT-mediated dUTP Nick End Labelling |
| TWEEN 20 | polyoxyethylene-sorbitan monolaurate |
| UCH-L1 | ubiquitin carboxyl-terminal-hydrolase L1 ( PGP9.5) |
| UPS | Ubiquitin-Proteasome System |
| UV | ultraviolet |
| VHS | virion host shut-off |
| VP16 | virion protein 16 |
| wt | wild type |
| X-gal | 4-Cl,5-bromo,3-indolyl-β-galactosidase |
| $\Delta\Psi m$ | mitochondrial membrane potential (Delta psi) |

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**CHAPTER 4**

# CHAPTER 1

# INTRODUCTION

# 1. Introduction

The work described in this thesis attempted to investigate the potentially toxic effects of Familial Amyotrophic Lateral Sclerosis (FALS) associated SOD1 mutants G93A and G93R and the protective effects of its wild-type form, in an *in vitro* cellular model. As a result of substantial experimental evidence on the protective effects of heat shock proteins (Hsps) it was decided to investigate and test their neuroprotection in this system and further investigate the incorporated mechanisms. For this purpose, Herpes Simplex Virus type 1 (HSV-1) based vectors, originally developed for gene therapy studies in the central nervous system, were utilised in order to efficiently over-express Hsps in mammalian neuronal cells.

Therefore the Introduction of this thesis focuses on the following aspects, which are directly relevant to the work presented here:

**a.** ALS disease genetics with reference to the SOD1 gene in hereditary ALS; the SOD multigene family; a detailed review on SOD1 gene in ALS and the experimental models of ALS along with other candidate genes that have been investigated; proposed toxic function of mutant SOD1.

**b.** The role of programmed cell death in FALS, an overview of apoptotic cell death.

**c.** Heat shock protein biology, their roles in the cell and in the context of neurodegeneration, as well as the role of Hsps as therapeutic targets in FALS and other neurodegenerative disorders. A brief account is also given of the viral vectors used in this study and their potential use in the development of gene therapeutic strategies for ALS and other diseases.

## 1.1 Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is an adult-onset, rapidly progressive and ultimately fatal neurodegenerative disorder. The disease, also referred to as motor neuron disease or Lou Gehrig's disease, is caused by a progressive loss of motor neurons in the

cortex, brainstem, and spinal cord (de Belleroche et al., 1995). On average the disease progresses for 3-5 years, leading to paralysis and premature death as a result of respiratory paralysis.

Approximately 10% of ALS cases are familial (FALS) (Camu et al., 1999), with the majority of cases considered to be sporadic (SALS) in the absence of any positive family history. The majority of FALS and SALS cases are clinically indistinguishable however there are some familial cases with clearly distinct features. Massive accumulation of neurofilaments is observed in motor neurons in both familial and sporadic ALS cases. Studies on animals have shown that motor neuron dysfunction precedes the onset of symptoms and that compensatory mechanisms are successful in maintaining motor functions until loss of motor units exceeds 50%, at which stage symptoms appear and rapid motor decline in the number of motor units occurs (Cote et al., 1993; Kennel et al., 1996). The progressive increase of the aged in the population is likely to be associated with a proportional increase of age-related disorders such as ALS (Riggs et al., 1992; Neilson et al., 1994; Lilienfeld et al., 1993; Durrleman et al., 1989).

ALS may present in three main ways, either as a predominantly lower motor neuron (LMN) form designated progressive muscular atrophy (PMA) or as a predominantly upper motor neuron (UMN) form called primary lateral sclerosis (PLS), but what is more commonly seen is a mixture of both UMN and LMN deficits. ALS usually begins focally and then spreads and involves both corticospinal (upper) and spinal (lower) motor neurons. Lower motor neuron involvement results in muscle denervation of the affected muscles, loss of tendon reflexes and fasciculation, and subsequently muscle atrophy. The degeneration of corticospinal motor neurons results in additional focal spasticity. Before the disease spreads, clinical experience suggests that it starts with fatigue, cramp, muscle weakness and wasting of one or more limbs or fasciculation of the tongue (bulbar onset). Loss of reflexes is not as common as pathologically increased reflexes (Swash, 1999). In addition, ALS may primarily involve the bulbar muscles where it is known as progressive bulbar palsy (PBP) or the limbs where it is called spinal ALS.

By reason of the progressive nature of the disease, the clinical picture may be incomplete at the onset of symptoms. The recently revised El Escorial system (Brooks, 1994) provides an outline for the criteria required for the diagnosis of suspected, possible, probable, and definite ALS based on clinical, electrophysiological, and neuropathological examination. The diagnosis of ALS requires the presence of both upper and lower motor neuron features with disease progression with the exclusion of any other disease that may account for these signs. A recent review reported that dementia is found in approximately 3-5% of ALS patients (Al Chalabi et al., 2000), however an earlier study had stated the figure as being as high as 15% (Hudson, 1981).

Even though the majority of sporadic and familial ALS cases are clinically identical, there are some minor features that distinguish the types, such as the average age of symptom onset; in SALS it is 56 years, whereas in FALS it is 46 years (Camu et al., 1999). There is no observed genetic anticipation. Overall, a male preponderance of 1.5:1 is reported worldwide, however this is not as pronounced in the >70 years age-group. The male preponderance only occurs in sporadic ALS and still remains unexplained (Haverkamp et al., 1995; Cashman et al., 1999). The duration of FALS is bimodal in that a small percentage of patients have a very poor prognosis, with an average survival of <2 years, while the remainder have a better prognosis than is observed in sporadic cases, with survival usually >5 years (Camu et al., 1999). Site of onset is variable, but bulbar onset is considered rare. Survival of individuals is not affected by age or gender, but instead by the site of symptom onset with longer survival being seen when the arms are affected first, compared to onset in the legs or oropharyngeal muscles (Mulder et al., 1986).

Despite more than a century of research, there is currently no cure for ALS. The drug riluzole is the primary form of treatment and acts by blocking glutamate release (Miller et al., 1996), although it has been demonstrated in rats that treatment with this drug may increase motor neuron survival by stimulating trophic activity in astrocytes (Peluffo et al., 1997).

The underlying cause of ALS still remains unknown. Although the familial form of ALS comprises only 10% of all cases, it is frequently used as the basis of study because of

the implicit genetic factors, with the aim of mapping and identifying gene(s) that trigger or predispose an individual to ALS. It is thought that identification of FALS genes may lead to a greater understanding of the mechanisms of cell death involved in all forms of ALS, which in turn, may lead to development of more effective treatments and therapies.

### 1.1.1 Epidemiology

The crude prevalence of ALS is estimated at being between 4-6/100,000 population and increases with age, peaking in the 60-75 years age-group at about 33/100,000 population and 14/100,000 population, for men and women respectively (Annegers et al., 1991; Traynor et al., 1999; Bobowick et al., 1973). The incidence rate of ALS is estimated at being between 1-3/100,000 person years and increases with age (Annegers et al., 1991; Traynor et al., 1999; McGuire et al., 1996) with a peak incidence rate being observed in the 55-75 years age-group of 10.5/100,000 for men and 7.4/100,000 for women, respectively. Ethnicity data from the United States (US) show lower rates of mortality from ALS among non-whites compared to whites, with a ratio of 1: 1.6, but exhibit age and gender differences similar to whites (Elian et al., 1993).

A latitude-related increase in ALS has been demonstrated, with geographic age-adjusted incidence rates ranging from 2.0 in Israel (32° latitude) to 8.0 in the northern Scandinavian countries (>60° latitude) (Kahana et al., 1984; Jokelainen, 1977; Tysnes et al., 1991; Gunnarsson et al., 1996). Geographical differences of ALS worldwide may probably be due to regional differences in disease diagnosis, regional differences in genetic susceptibility and/or exposure to environmental factors. Four geographic areas with a high prevalence of ALS (clusters) are known and have been well-studied. In the western Pacific islands of Guam and Rota, the Chamorro population commonly suffer from two neurologic conditions; one is ALS, locally known as 'lytico'; and the other parkinsonism-dementia complex (PDC) of Guam, locally known as 'bodig'. Lytico and bodig can occur together in patients and families (Kurland, 1954), although the strong notion of an identical etiology has as yet not been proven (Oyanagi et al., 1999). The crude prevalence of ALS on Guam has fallen in the 1950s from 100/100,000 population, to 50/100,000 for men and 25/100,000 population for women, in the 1990s (McGeer et al., 1997; Wiederholt et al., 1999; Zhang et

al., 1996). A similarly high prevalence of ALS is shown in two areas of the Japanese Kii peninsula (Yase et al., 1968; Handa et al., 1963), and also by a population of the western coast of former West Papua New Guinea, now Irian Jaya (Indonesia) (Gajdusek et al., 1982). A fourth high-incidence area is found at the same pacific latitude, in the Gulf of Carpentaria (North Australia), in an isolated tribe at Anguru on Groote Eylandt (Kiloh et al., 1980). In these South Pacific cluster areas, ALS is said to account for about one in 10 deaths. In addition, an unusually high frequency of atypical Parkinsonism occurs in Guadeloupe (in the French West Indies), which is occasionally accompanied by ALS, reminiscent of that described in Guam (Chen et al., 1996). The potential reasons for these clusters are discussed below.

Smaller clusters of ALS, which are linked by professional or residential proximity, have been reported, for instance, from the same apartment building (n = 3) (Mekned et al., 1982), a school (n = 3) (Hyser et al., 1987), a group of farm-rangers in South-Dakota (n = 4) (Hochberg et al., 1974; Kilness et al., 1977; Dal Canto et al., 1994.), as well as among mail carriers (n = 3) in a small town (Sanders et al., 1980), and also a small community (six cases of ALS between 1975 and 1983) near lake Michigan, Wisconsin, USA (Sienko et al., 1990; Taylor et al., 1989). In addition to being reported in Guam (Reed et al., 1975), conjugal ALS has also been reported in four couples outside the Western Pacific (Cornblath et al., 1993; Maloo et al., 1987; Chad et al., 1982; Paolino et al., 1983). These ALS clusters may be the result of a common exposure or transmission of a causal agent, or alternatively they may be purely coincidental. The analysis of small clusters is usually complicated due to the lack of data on the total population of origin.

### 1.1.2 Environmental risk factors

Until recently, the environmental risk factors involved in neurodegeneration in ALS were largely unknown. In addition, genetic factors could not fully explain the endemic occurrence of ALS in the high-risk areas, such as in the Pacific (Bailey-Wilson et al., 1993; Ahlskog et al., 1995), and as a result evoked an animated discussion on gene-environment interactions. Cycad nuts, along with mineral content of the drinking water and also soil content were all suspected to be long-term environmental neurotoxic risks. Above all, atten-

tion focused especially on the fruits of the locally growing cycad palms (Kisby et al., 1992; Spencer et al., 1991; Duncan, 1992), because of the fact that geographic incidence rates for ALS are strongly correlated with concentrations of cycasin in traditional food made from the toxic seed of the cycad plants (Zhang et al., 1996; Kisby et al., 1992). The pathogenic effect of cycasin appears to be a decrease of neuronal DNA repair, along with persistent up-regulation of Tau mRNA expression and also enhancement of excitatory neurotoxicity (Esclaire et al., 1999).

Other alleged environmental risk factors for ALS include a history of trauma to the brain and spinal cord, exposure to radiation, electrical shocks, strenuous physical activity, welding or soldering materials, as well as employment in petroleum, paint or dairy industries. However, there have not been any consistent reports of the exogenous risk factors for ALS. Although, in a population study, malignancies were reported in 10% of patients with motor neuron disease (Gubbay et al., 1985) but this association still remains unexplained. Another potential etiology of ALS is thought to be long-latency viral infection (Giraud et al., 2000; Karpati et al., 2000). A French study using reverse transcription-polymerase chain reaction (RT-PCR), found enterovirus nucleic acids in 15/ 17 spinal cords of sporadic ALS patients (Berger et al., 2000; Woodall et al., 1994; Muir et al., 1996; Swanson et al., 1995; Walker et al., 2001), but in no more than 20 spinal cords examined in other studies (Berger et al., 2000; Woodall et al., 1994; Muir et al., 1996; Swanson et al., 1995; Walker et al., 2001).

In conclusion, there is inconsistency in exogenous risk factors reported in ALS, which might imply that these risk factors do not, as such, cause the pathology. Alternatively, it might reflect a complex interaction between a number of environmental factors and specific genetic susceptibilities.

### 1.1.3 Genetic Risk Factors

Extensive research has been carried out into the genetic risk factors associated with ALS. Familial ALS (FALS) is clinically and genetically heterogeneous and has multiple autosomal-dominant and recessive forms. Genetic analysis of FALS has implicated the

involvement of several genes in ALS (Table 1.1; references are not included in the table but are cited in the text when the gene is mentioned). For the purposes of this thesis, these genes will be crudely divided into two groups "major genes" and "susceptibility genes". The genes referred to as "major genes" cause ALS with a clearly monogenic inheritance pattern. They may either predominantly lead to ALS (ALS1-ALS6) or result in multisystem neurodegeneration (tauopathies and ALS with dementia and Parkinsonism) with ALS occurring as an occasional symptom.

Those referred to as "susceptibility genes" are the genes, which may trigger the cascade of neurodegeneration or alternatively act as susceptibility factors for neurodegeneration in concurrence with environmental risk factors or other (genetic) risk factors.

**Table 1.1 Genetics of amyotrophic lateral sclerosis (ALS)**

| Classification | Gene | Localization | Inheritance |
|---|---|---|---|
| **Major genes** | | | |
| ALS1 | SOD1 | 21q22 | AD/AR |
| ALS2 | ALSin | 2q33-34 | AR |
| ALS3 | | Unknown | AD |
| ALS4 | | 9q34 | AD |
| ALS5 | | 15q 12-21 | AR |
| ALS6 | | 18q21 | AD |
| FTDP | Tau | 17q | AD |
| FTD | | 9q21-22 | AD |
| **Susceptibility genes** | | | |
| Neurofilament heavy chain | NF-H | 22q12.2 | |
| Neurofilament light chain | NF-L | 8p21 | |
| Peripherin | PRPH | 12q12-13 | |
| Glutamate transporter | EAAT2 | 11p13-12 | |
| Glutamate receptor | AMPA | 5p33 | |
| Apolipoprotein E | ApoE | 19q13.2 | |
| Ciliary neurotrophic factor | CNTF | 11q12.2 | |
| Debrisoquine hydroxylase | CYP2D | 22q13.1 | |
| Apurinic apyrimidinic | APEX | 14q11-12 | |
| Mitochondrial DNA | COX | | |
| Manganese superoxide dismutase | SOD2 | 6q25 | |
| P2 blood group | P2 | 22q11 | |

**Abbreviations:** AD, autosomal dominant; AR, autosomal recessive

### 1.1.4 Major genes

I will first discuss the monogenic forms of ALS, which show clear Mendelian inheritance. Seven different gene loci have been reported for FALS, and three major genes (SOD1, ALSin and Tau) have been identified (Table 1.1).

The difficulty of clinically distinguishing ALS from other forms of motor neuron disorders, particularly in patients who do not exhibit the classical signs of each disease, is highlighted by the occasional finding of a deletion in one of the genes for spinal muscular atrophy, the survival motor neuron gene SMN1 (Corcia et al., 2002), centromeric SMN2 (Veldink et al., 2001) and the neuronal apoptosis inhibitory protein gene, NAIP, in patients diagnosed with sporadic and familial ALS (Parboosingh et al., 1997; Orrell et al., 1997; Moulard et al., 1998; Jackson et al., 1996). Additionally, ≈2% of male patients diagnosed with ALS, in fact have Kennedy disease (SBMA, X-linked spinobulbar muscular atrophy) brought about by a trinucleotide expansion in the androgen receptor (Garofalo et al., 1993; Parboosingh et al., 1997).

Autosomal-dominant inheritance of FALS transpires in approximately 5-10% of patients suffering from ALS and is clinically and neuropathologically indistinguishable from sporadic ALS (Cudkowicz et al., 1997). Dominant FALS is characterized by large intra- and interfamilial variability of both age of onset and disease progression. In some pedigrees, incomplete penetrance has been observed (de Belleroche et al., 1995; Chio et al., 1987; Husquinet et al., 1980; Suthers et al., 1994) and confirmed by mutation analysis (Cudkowicz et al., 1998).

Autosomal-dominant FALS is genetically heterogeneous and currently two genes, one on chromosome 21 (SODl) and the other on chromosome 17 (TAU) as well as three additional loci (one on chromosome 18 and two on chromosome 9), are known (Table 1.1).

Before linkage analysis and molecular diagnosis of ALS became available through molecular analysis, autosomal-recessive FALS was deemed very rare. Nevertheless, three forms of recessive ALS have already been recognized, and the genes for two of those, SOD1

(ALS1) and ALSin (ALS2) have now been identified. Although in the majority of cases, SOD1 occurs as a dominant mutation, there are a small number of SOD1 mutations, which are recessive as discussed later in this chapter. In total these established forms of FALS probably account for approximately 20-30% of all FALS cases.

### 1.1.4.1 ALS1 (SOD1)

In 1991 the first ALS gene (ALS1), for an autosomal-dominant form of FALS, was mapped to chromosome 21q (Siddique et al., 1991) and subsequently shown to be the cytosolic copper-zinc superoxide dismutase (SODl) gene two years later (Rosen et al., 1993). Therefore, SOD1 is a major gene and an important locus for autosomal dominant FALS and is the focus of this thesis; it will be discussed in greater detail later, after a brief overview of all the other major loci.

### 1.1.4.2 ALS2 (ALSIN)

Autosomal-recessive FALS was initially described in a large inbred family of several consanguineous matings in Tunisia showing linkage to the chromosomal region 2q33-34. Symptoms occur in the first or second decade of life and consist of progressive spasticity of the limbs as well as the facial and pharyngeal muscles with individuals displaying a relatively long survival of approximately 15 to 20 years (Hentati et al., 1994).

The gene for ALS2 was recently identified in Tunisian and Kuwaiti families and named alsin or als2 (Hadano et al., 2001; Yang et al., 2001). Alternative splicing of this gene generates two transcripts one short and one long. Deletions affecting both transcripts give rise to the ALS2 phenotype, while homozygous deletions in exon 9 and affecting only the short form of the protein, cause a form known as juvenile primary lateral sclerosis (JPLS). JPLS is even rarer than ALS2 and its phenotypic presentation involves slowly progressive spastic paraparesis and muscle weakness of the oro-facial and ocular muscles.

Expression of the mouse ortholog of alsin was detected in neuronal cells throughout the brain and spinal cord. Alsin has protein domains with similarities to both pleckstrin and

guanine-nucleotide exchange factors (GEFs) known to activate GTPases and therefore may act as a regulator/activator of GTPases, and modulate microtubule assembly, membrane organization and trafficking in neurons (Majoor-Krakauer et al., 2003).

#### 1.1.4.3 ALS3

The majority of dominant FALS has not yet been assigned to any given locus and as a result is given the designation of ALS3 (Siddique et al., 1991).

#### 1.1.4.4 ALS4

A second form of autosomal-dominant FALS (ALS4) has been localized to a locus on chromosome 9q34 in an extended family with juvenile onset. The mean age of onset is 17 years and the disease has slow progression without any bulbar involvement. As yet the ALS4 gene has not been identified. At the outset, patients show difficulty in walking, with subsequent weakness and wasting of muscles of the hand and distal lower extremities; significant proximal weakness takes place in the fourth or fifth decade of life and loss of useful hand function by the sixth decade. Neuropathological examination revealed degeneration extending to the dorsal roots, dentate nucleus, nucleus gracilis, and inferior olivary nucleus (Rabin et al., 1999).

#### 1.1.4.5 ALS5

This form of autosomal-recessive FALS possesses clinical similarities to ALS2 (Hentati et al., 1997). ALS5 is thought to be the most prevalent form of recessive ALS, and was identified in several ethnic groups such as North African, South Asian, and European. The ALS5 gene was mapped to chromosome 15q, but as yet remains to be identified (Cox et al., 2000).

### 1.1.4.6 ALS6

Recently, a third locus for autosomal-dominant FALS was mapped to chromosome 18q21 in a large European family exhibiting classic adult-onset ALS (Hand et al., 2001).

### 1.1.4.7 Tauopathies

ALS1, ALS4 and ALS6 all represent pure forms of autosomal-dominant FALS. However, ALS can additionally be part of a multisystem neurodegeneration comprising the Tauopathies and the combination of ALS with dementia and Parkinsonism. Mutations in the tau gene are associated with a clinical phenotype that incorporates frontotemporal dementia, Pick's disease, corticobasal degeneration, and familial progressive supranuclear palsy. This phenotype exhibits variability both within and between different families (Wilhelmsen et al., 1994; Stanford et al., 2000; Bird et al., 1999; Spillantini et al., 2000; Bugiani et al., 1999; Pastor et al., 2001). The variability of clinical symptoms implies that tau pathology may play a part in a large number of neurodegenerative diseases, including ALS, where neurofilamentous deposits consisting principally of hyperphosphorylated Tau protein transpire. Tau mutations were first recognized in a syndrome known as frontotemporal dementia and Parkinsonism complex (FTDP) (Wilhelmsen et al., 1994; Hutton et al., 1998), characterized by autosomal-dominant inheritance of behavioural disturbances, reduced speech and memory impairment; with the possibility of Parkinsonism and amyotrophy occurring later during the course of the disease. The prevalence of FTDP in the 60-70years age-group is estimated to be about 2.8 per 100,000 population (Stevens et al., 1998). The proportion of families with FTDP also possessing a mutation in the tau gene varies from approximately 13% to 40% (Houlden et al., 1999; Rizzu et al., 1999; Poorkaj et al., 2001).

In FTDP, mutations of the tau gene have an effect on the alternative splicing of exon 10 and as a result lead to an excess of four-repeat Tau isoforms. The four-repeat protein Tau isoforms may lead to neurodegeneration through reduced binding of Tau to microtubules in axons. This might explain the accumulation of Tau protein as hyper-phosphorylated neurofilaments in the cytoplasm, which results in central and peripheral axonopathy and

triggers amyotrophy and neuronal death (Heutink, 2000; Ishihara et al., 1999; Probst et al., 2000; Goedert et al., 1998).

Guadeloupean Parkinsonism associated with frontolimbic dementia and ALS, is also regarded as a tauopathy as the result of the accumulation of Tau proteins, predominantly in the midbrain. While no mutations in the tau gene were observed in this group of patients, all cases were homozygous for the H1 tau haplotype (Caparros-Lefebvre et al., 2002). The Tau polymorphism CA3663 is associated with the ALS, dementia-parkinsonism complex of Guam, which indicates that Tau is not the cause of the disease but possibly acts as a susceptibility gene (Schmidt et al., 2001; Poorkaj et al., 2001).

### 1.1.4.8 ALS with dementia and Parkinsonism

The clinical phenotype of FALS can be comprised of pure ALS, multisystem degeneration in the tauopathies, and ALS in association with dementia and Parkinsonism. Some mutations in SOD1 may trigger ALS associated with dementia, as observed in a family with the A76T mutation (Andersen et al., 1997). Parkinsonism is observed less frequently, suggesting reduced toxicity of SOD1 mutations to nigrostriatal dopaminergic neurons (Przedborski et al., 1996). The type of ALS, which occurs in Guam, is characterized by the familial co-occurrence of dementia, Parkinsonism and motor neuron signs. Analysis of the SOD1 gene in this disease uncovered an I113T mutation in (two of 23 unrelated) ALS patients from the Kii Peninsula of Japan (Kikugawa et al., 1997; Figlewicz et al., 1994).

Other genes predisposing to age-related multisystem neurodegeneration also symbolize a genetic contribution to ALS. Mutations in the tau gene result in varying symptomatology, as recently demonstrated in the FTDP (Wilhelmsen et al., 1994; Bird et al., 1999; Hutton et al., 1998; Clark et al., 1998; Dumanchin et al., 1998) and in familial atypical progressive supranuclear palsy (Stanford et al., 2000), while the ALS-frontotemporal dementia complex (FTD) is linked to chromosome 9q (Table 1) (Hosler et al., 2000). Nevertheless, these genes do not account for all of the familial aggregation of ALS, dementia and Parkinsonism (Ludolph et al., 1992; Rossor et al., 2000).

Epidemiologic studies have revealed familial aggregation of classic ALS, dementia and Parkinsonism, suggesting that all three disorders may have common genetic factors. A significant increase in the risk for dementia and also for Parkinsonism, in first- and second-degree relatives of ALS patients has been observed in a number of case-control studies (Haverkamp et al., 1995; Majoor-Krakauer et al., 1994; Deapen et al., 1986; Gunnarsson et al., 1992). The cumulative incidence of dementia to 90years of age was significantly higher, approximately 15%, in relatives of ALS patients than in relatives of controls. In addition, the lifetime risk for contraction of Parkinsonism is 7% for relatives of ALS patients, a two-fold increase compared to relatives of controls (Majoor-Krakauer et al., 1994). Epidemiologic data also revealed an excess of familial co-occurrence of dementia and Parkinsonism in families of patients suffering from ALS; with patients with sporadic ALS almost 12 times more likely to have relatives with Parkinsonism, if a family member had dementia than if there was no family history of dementia (Majoor-Krakauer et al., 2003). This excess of co-occurrence probably indicates a genetic susceptibility to widespread neurodegeneration.

Aside from the four loci described above, there are many more genes involved in syndromes with ALS, dementia and/or Parkinsonism. Machado-Joseph disease exhibits signs of familial amyotrophy associated with Parkinsonism and spinocerebellar symptoms, and is brought about by expansion of trinucleotide repeats in the SCA3 gene on chromosome 14. Other examples of genetic disorders presenting with both pyramidal and extra-pyramidal symptoms are familial aggregation of motor neuron disease, tic disorder and parkinsonism triggered by neuroacanthosis (Vance et al., 1987; Spitz et al., 1985; Rubio et al., 1997), and the autosomal-dominant inherited combination of parkinsonism and amyotrophy (Ziegler et al., 1972; Brait et al., 1973; Alter et al., 1976; Tranchant et al., 1992). A familial ALS-dementia complex of autosomal-recessive inheritance was reported in some families, including inbred Old Amish sib-ships. The latter type of ALS, is a juvenile form of ALS with an exceptionally long survival ranging from 9 to 27 years, and is variably accompanied by dementia (Staal et al., 1968; Otero Siliceo et al., 1998). Another familial ALS-dementia complex exhibits an autosomal-dominant pattern of inheritance (Dazzi et al., 1969; Finlayson et al., 1973; Pinsky et al., 1975; Gunnarsson et al., 1991). The familial occurrence of ALS with symptoms of dementia, Parkinsonism, or other central nervous system (CNS) symptoms is also observed in an increasing number of different genetic

multisystem neurodegenerative disorders, such as Huntington's disease, or neuroaxonal dystrophy (Rosenberg, 1982; Bots et al., 1973). Hence, family histories of patients with ALS should be investigated in detail, not only for ALS, but also for the other often-related disorders of dementia and Parkinsonism.

## 1.2 Structure, Evolution and Expression of Superoxide Dismutase CuZn-SOD (SOD1)

The evolution of aerobic organisms that can survive in oxygen-rich environments necessitates an effective defence system against reactive oxygen species (ROS), which are generated following single electron reductions of molecular oxygen. While physiological concentrations of ROS in aerobic organisms are advantageous and involved in cell signalling pathways and survival from invading pathogens, an unbalanced, elevated concentration of ROS may play a role in the development of various diseases, such as atherosclerosis, cancer, diabetes, hypertension, inflammation, and premature aging.

The superoxide dismutases (SODs) are the principal and most significant line of antioxidant enzyme defence systems against ROS and particularly the superoxide anion radicals. Three distinct isoforms of SOD have been observed in mammals, and their genomic structure, cDNA, and proteins have been described. Two of the isoforms of SOD have $Cu^{2+}$ and $Zn^{2+}$ in their catalytic centre and are localized to either intracellular cytoplasmic compartment (CuZn-SOD or SOD1) or to the extracellular environment (EC-SOD or SOD3). SOD1 has a molecular mass of approximately 32,000 Da and is present in the cytoplasm, nuclear compartments, and lysosomes of mammalian cells (Chang et al., 1988; Keller et al., 1991; Crapo et al., 1992; Liou et al., 1993). SOD3 is the most recently discovered and least characterized member of the SOD family and exists as a homotetramer of molecular weight 135,000 Da with high affinity for heparin (Marklund et al., 1982). SOD3 was initially detected in human plasma, lymph, ascites, and cerebrospinal fluids (Marklund et al., 1982 & 1986). The pattern of expression of SOD3 is highly restricted to these specific cell type and tissues where its activity can surpass that of SOD1 and SOD2. The last isoform of SOD has manganese (Mn) as a cofactor and is localized to mitochondria

of aerobic cells (Mn-SOD or SOD2) (Weisiger et al., 1973) where it exists as a homotetramer with an individual subunit molecular weight of about 23,000 Da (Barra et al., 1984). SOD2 has been shown to play a role in promoting cellular differentiation and tumorgenesis (St. Clair et al., 1994) and in shielding against hyperoxia-induced pulmonary toxicity (Wispe et al., 1992).

### 1.2.1 Evolution of SOD

The appearance of SOD enzymes was sparked by the proliferation of photosynthetic organisms that started to produce oxygen about 2 billion years ago. A range of antioxidant enzymes evolved to offset the toxic effects of by-products of oxygen metabolism. At this time two major types of superoxide dismutase appeared in prokaryotes as a result, copper/zinc-containing SODs and iron/manganese-containing SODs. Although both types of enzymes perform the same function, a strong argument against a common ancestor comes from their completely different crystal structures, use of different metal cofactors, and distinctive catalytic mechanism. The evolutionary tree for CuZn containing SOD, based on multiple sequence alignments with structural superimpositions of crystal structures, shows that at early stages of evolution, before the differentiation of fungi, plants, and metazoa, extracellular SOD diverged from the cytosolic form (Bordo et al., 1994). The SOD1 structural core exists as a Greek key β-barrel motif, consisting of eight β-barrels (Getzoff et al., 1989). Amino acid substitutions, insertions and deletions, predominantly occur outside of this structural motif. The theory that CuZn-SOD evolution involved gene duplication and fusion followed by addition of exons I and III is supported by these data. The evolutionary rates of CuZn- and Mn-SOD during the last billion years have differed considerably. Mn-SOD proteins have evolved at a relatively constant rate compared to CuZn-SODs, which initially evolved unusually slowly and then extremely erratically in the past 100 million years (Smith et al., 1992; Rodriguez-Trelles et al., 2001). Why such a peculiar evolutionary rate occurred still remains unclear; one possible explanation put forward suggests that CuZn-SOD was trapped in a "folding-block" with most changes in amino acid composition being deleterious (Smith et al., 1992). The accumulation of silent mutations ultimately led to an escape from this "evolutionary hibernation" and a return to the faster evolutionary rate. While the plausibility of this theory remains dubious, the existence of aerobic life on Earth

provides evidence that SOD successfully evolved as a potent protective enzyme against oxygen toxicity.

### 1.2.2 SOD1 Gene Structure

The genomic sequence for SOD1 has been identified in the mouse (Benedetto et al., 1991), rat (Kim et al., 1993; Hsu et al., 1992) and also human (Levanon et al., 1985). The SOD1 gene possesses five exons and four introns and exhibits striking similarity among species (Figure 1.1). The TATA and CCAAT boxes, along with several highly conserved GC-rich regions, have been localized in all three species with a similar pattern in the proximal promoter region. This high level of homology in the 5'-flanking sequence implies that evolutionary factors have preserved key regulatory regions for this gene. The 3' end of SOD1 gene contains several poly(A) signal sequences that result in termination and hence generation of mRNA species with differing lengths. The consensus sequences GTGTT and a G/T cluster necessary for efficient formation of 3'-termini have also been located in the rat SOD1 gene, downstream from the polyadenylation signal. In addition, several putative binding sites for NF1, Sp1, AP1, AP2, GRE, HSF, and NF-κB transcription factors have been found from studies of the promoter region of the human SOD1 gene (Kim et al., 1994). The role of the transcription factors Sp1 and Egr-1 has been confirmed respectively in basal and inducible expression of human SOD1 (Minc et al., 1999).

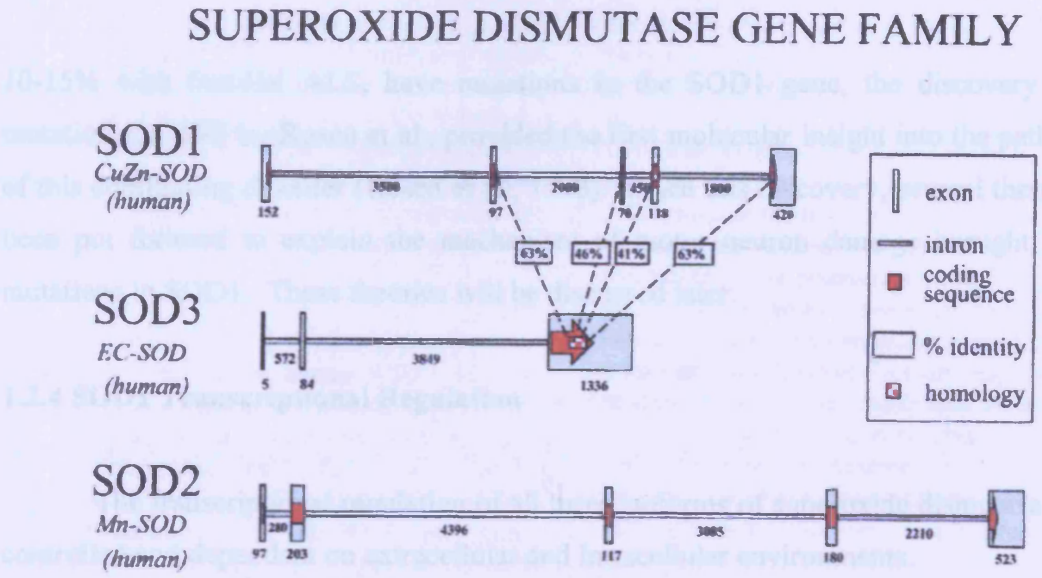



**Figure 1.1**. The genomic organization of the three members of the human SOD gene family. SOD3 is placed in the middle in order to demonstrate areas of amino acid sequence homology between SODl and SOD3. No significant amino acid sequence homology exists between SOD2 and either SODl or SOD3. The size of each exon and intron is shown in base pairs, in association with the respective fragment. The figure was adapted from Zelko et al., (2002).

### 1.2.3 SOD1 Chromosomal Localization and Polymorphisms

The SOD1 gene has been localized in humans to chromosome 21 (region 21q22.1) (Levanon et al., 1985), in bovine species to chromosome 1 (1q12-14) (Schmutz et al., 1996), and in the mouse to chromosome 16 (region 16B4-ter) (Francke et al., 1979). Human chromosome 21 has been intensively investigated due to the association between Down's syndrome and trisomy 21. Although patients with Down's syndrome exhibit an approximately 50% increase in SOD1 activity as a result of higher levels of SOD1 protein, the role of the enzyme in pathology associated with the disease remains uncertain. The increased dosage of SOD1 gene correlates with some symptoms of Down's syndrome, such as the pathological abnormalities of tongue neuromuscular junctions (Avraham et al., 1988; Groner et al., 1994) but cannot totally account for the development of the major symptoms (De La Torre et al., 1996). In contrast, more than 90 different mutations in the SOD1 gene have been linked to ALS. Although only a small proportion of patients, 2% with ALS and

10-15% with familial ALS, have mutations in the SOD1 gene, the discovery of these mutations in 1993 by Rosen et al., provided the first molecular insight into the pathogenesis of this debilitating disorder (Rosen et al., 1993). Since this discovery, several theories have been put forward to explain the mechanism of motor neuron damage brought about by mutations in SOD1. These theories will be discussed later.

### 1.2.4 SOD1 Transcriptional Regulation

The transcriptional regulation of all three isoforms of superoxide dismutase is highly controlled and dependent on extracellular and intracellular environments.

SOD1 has a widespread distribution in a variety of cells (Crapo et al., 1992). Cytoplasmic SOD1 expression is stable and its activity is frequently considered as an internal control for SOD1 gene expression.

### 1.2.5 Stimuli upregulating SOD1 expression

Although SOD1 is considered to be constitutively expressed, various physiological conditions can dramatically regulate its mRNA levels. A wide array of mechanical, chemical, and biological messengers such as heat shock (Hass et al., 1988; Yoo et al., 1999), shear stress (Inoue et al., 1996; Dimmeler et al., 1999), UVB- and X-irradiation (Isoherranen et al., 1997; Leecia et al., 2001; Yamaoka et al., 1994), heavy metals (Yoo et al., 1999), hydrogen peroxide (Yoo et al., 1999), ozone (Rahman et al., 1991), nitric oxide (Frank et al., 2000), arachidonic acid (Yoo et al., 1999), and xenochemicals such as β-naphthoflavone, t-butyl-hydroquinone, iodoacetamide (Yoo et al., 1999), 2,3,7,8-tetrachlorodibenzo-p-dioxin (Cho et al., 2001), and phenobarbital (Ueda et al., 2002) result in elevation of SOD1 mRNA levels. Proximal promoter region analysis reveals Spl/Egr-1/WT-1 binding sites that are involved in basal and TPA inducible expression of SOD1 (Minc et al., 1999) in addition to C/EBP cis-acting elements (Seo et al., 1996 & 1997), which are important for high-level expression in rat liver cells (Kim et al., 1997). SOD1 expression can also be prompted by ginseng saponins through activation of the transcription factor AP2 (Kim et al., 1996). Inside cells, metal ions are a potent source for the large-scale catalysis and generation of

ROS. In order to neutralize their harmful effects, the synthesis of SOD1 in the cell is increased through the metal responsive element located in the 5'-flanking region (Yoo et al., 1999).

### 1.2.6 Stimuli downregulating SOD1 expression

SOD1 downregulation has been shown in alveolar type II epithelial cells and lung fibroblasts following exposure to hypoxia (Jackson et al., 1996). Mitomycin C, an anticancer drug, also suppresses the transcription of SOD1 gene in human hepatoma HepG2 cells (Cho et al., 1997).

### 1.2.7 SOD1 Structure and Activities

Genetic analysis of Familial ALS has yielded seven loci and one disease gene SOD1 as mentioned previously. Initially a role for free radicals was suggested in the disease process, although the mechanism by which the mutant brings about toxicity remains uncertain. Oxygen free radicals such as the superoxide anion ($O^{2-}$), the hydroxyl radical ($^{\cdot}OH$), and other reactive oxygen species such as hydrogen peroxide ($H_2O_2$) and nitric oxide (NO), are generated in the cell by a number of metabolic reactions (Fullerton et al., 1998). The most important defence against the production of excessive free radicals is the collective action of three enzymes: superoxide dismutase (SOD), glutathione peroxidase, and catalase. SOD catalyses the conversion of superoxide to $H_2O_2$ and glutathione peroxidase and catalase then convert $H_2O_2$ to water (Brown, 1997). However, in the presence of NO, superoxide can form peroxynitrite ($ONOO^-$), which can subsequently react to produce hydroxyl radicals. Superoxide is produced as a result of normal cellular metabolism and is extremely toxic, disrupting cellular activity by attacking membrane lipids, proteins, and DNA (Fullerton et al., 1998).

Prior to its association with FALS, SOD1 had been intensively investigated due to its biological importance as an antioxidant. SOD1 is an abundant protein comprising approximately 1% of total cytosolic protein (Bredesen et al., 1996) and is constitutively expressed in all cells in eukaryotes and highly conserved through evolution (Brown, 1997).

The gene for human SOD1 is located on chromosome 21q22.1, spanning approximately 11 kb of DNA (Andersen, 1997). It has five exons and yields a 153-amino-acid, 16-kDa protein that functions as a homodimer.

Each subunit holds one atom of both $Cu^{2+}$ and $Zn^{2+}$ within what is described as a cave-like active site (Siddique et al., 1996). The polypeptide chain is folded into a flattened cylinder of eight strands of anti-parallel β-structure, which are arranged in two interlocking Greek key motifs forming the β-barrel. Two large non-helical loops extend from both the top and bottom of the β-barrel, with one containing most of the residues necessary for electrostatic guidance of $O^{2-}$, and other contributing to the dimer interface. The active site is located at one end of the β-barrel, with an opening facing out and away from the β-barrel and away from the other subunit, so the active sites are positioned in such a way that they are at opposite sides of the enzyme. The SOD1 dimer is extremely stable as a result of strong hydrophobic interactions and in addition the dimerization doubles the dismutase activity. Superoxide is steered toward the active site containing the $Cu^{2+}$ atom through a positively charged channel (Siddique et al., 1996). The $Zn^{2+}$ is involved in maintaining pH stability of the dismutation reaction and also in the speedy dissociation of the $H_2O_2$ generated, which can slowly inactivate SOD1 (Andersen, 1997). Access to the active site is limited by size and charge of the molecules, favouring $O^{2-}$ and excluding other larger molecules (Siddique et al., 1996). In addition to dismutase activity, SOD1 has a peroxidase function, catalysing oxidative reactions of substrates by hydrogen peroxide.

## 1.3 ALS Mutations of SOD1

An initial study of 13 autosomal dominant ALS families, carried out to establish a causal link between the SOD1 gene and FALS, identified a total of 11 missense mutations in two exons (Rosen et al., 1993). This discovery resulted in an explosion of SOD1 gene screening in ALS pedigrees. At present, more than 90 different mutations occurring in all five exons of this gene have been identified (Cudkowicz et al., 1997). There is a database that lists all the SOD1 ALS mutations described (www.alsod.org). All of these mutations in SOD1 are associated with the autosomal-dominant form of FALS, with the exception of two

- D90A and D96N - which can cause both dominant and recessive ALS depending on the ancestry of the individual concerned; those individuals of Scandinavian ancestry with D90A and D96N SOD1 mutations generally display the recessive form of the disease whereas others display the dominant form (Andersen et al., 1995; Robberecht et al., 1996; Orrell, 2000; Hand et al., 2001).

SOD1 mutations have been detected in 195/916 families exhibiting the autosomal-dominant form of ALS, suggesting that SOD1 is implicated in ± 21% (range 13-84%) of dominant FALS cases (Cudkowicz et al., 1997; Rosen et al., 1993; Jones et al., 1995; Pramatarova et al., 1994; Andersen et al., 1997; Juneja et al., 1997; Orrell et al., 1997 & 1999; Aguirre et al., 1999). Additionally, 112 of 946 patients (or about 12%, range 2.5-23%) with apparently sporadic ALS were heterozygous for a SOD1 mutation (n=112) (Andersen et al., 1996; Cudkowicz et al., 1997; Jackson et al., 1997; Orrell et al., 1997). The mutation pattern in sporadic ALS cases is similar to that observed in FALS cases (Radunovic et al., 1996; Hayward et al., 1996). Sporadic ALS, which results as a consequence of a SOD1 mutation, may represent a new dominant mutation, or incomplete penetrance of a dominant mutation in the parents. A number of the SOD1 mutations occur as recurrent mutations or as founder mutations, occasionally having a worldwide distribution.

One confirmed autosomal recessive mutation, which has been documented, is the D90A mutation in exon 4. This missense mutation, involving an A to C transversion, results in the substitution of aspartic acid for alanine at codon 90, and can cause ALS, both in the homozygous state or in the compound heterozygous state with the D96N mutation (Hand et al., 2001; Mezei et al., 1999). The recessive inheritance of the D90A mutation has been documented largely in Scandinavian families (Andersen et al., 1995, 1996 & 1997). In other parts of the world, the D90A mutation is not a common cause of recessive FALS and in the heterozygous state typically results in the dominant form of ALS (Robberecht et al., 1996; Aguirre et al., 1999; Jackson et al., 1997; Radunovic et al., 1996; Khoris et al., 2000; Morita et al., 1996). Homozygotes for this mutation, in the Northern Scandinavian population, exhibit a uniform disease phenotype with insidious onset, mean age of onset of 44 years, ranging from 20-94. The slowly ascending paresis commences distally in the lower extremities and has relatively slow progression with a mean survival of about 14 years)

(Aguirre et al., 1999; Andersen et al., 1996). In the population of northern Sweden and Finland, this type of ALS1 accounts for 9.6% (44/451) of ALS cases (Andersen et al., 1997; Sjalander et al., 1995), in addition a 10-fold increase in the allele frequency of the D90A mutation was found (1-2%) in this population (Andersen et al., 1996; Harris et al., 1974). A study of 28 recessive D90A pedigrees demonstrated that 20 families shared the same founder haplotype (Al-Chalabi et al., 1998), while a number of founders were detected in eight families with the dominant D90A mutation. Hence, it was proposed that there may be a protective factor that is tightly linked to the mutation in SOD1 in the genetically, rather homogenous, northern Scandinavian population which reduces susceptibility to ALS; this would also explain the very long disease duration in the Scandinavian patients.

Hand et al., (2001) also reported an ALS family in which affected individuals carried both a single copy of the SOD1 D90A recessive mutation and also a copy of a novel mutation D96N; this is the first description of compound heterozygosity in ALS and SOD1. The D96N mutation has been proposed to be another recessive mutation (Hand et al., 2001). An affected individual homozygous for the L84F mutation has been described, however, as yet, no further evidence exists that indicates this is a recessive mutation (Boukaftane et al., 1998).

So far, of the alterations identified in the SOD1 gene, the majority are base substitutions resulting in missense mutations. Three exonic insertions (Andersen et al., 1997; Jackson et al., 1997; Kato et al., 1996) and one exonic deletion (Pramatarova et al., 1994), which result in premature termination of the protein, have been described. Structurally, the earliest of these alterations takes place at the last codon of exon 4 (Jackson et al., 1997), with others lying within exon 5, so even though the protein is truncated only the last exon is lost. Importantly, exon 3, which encodes the active site, remains unaffected. Besides the ALS associated mutations, a number of variants and polymorphisms have been identified (Orrell, 2000). Initially, it was speculated as a result of the failure to detect mutations in exon 3, that as it encoded the active site, it was privileged from mutations and, so far, only three have been reported (Andersen et al., 1997; Boukaftane et al., 1998; Orrell et al., 1997). Only approximately 10-20% of ALS families carry SOD1 mutations (Camu et al., 1999), which reflects existence of families that do not link to chromosome 21 or ALS3

locus. Mutations have also been detected in sporadic ALS cases; however it is not clear as to whether all of these are truly sporadic. No mutations have been detected in individuals with the form of ALS seen in the Chamorro people of Guam (Figlewicz et al., 1994).

Recent investigations have focused on the question of which is the common denominator, the toxic function common to so many different mutant proteins - if indeed such a common denominator actually exists. Theoretical analysis based exclusively on molecular models predicts that biochemical properties of the mutant enzymes would be either affected dramatically or only secondarily depending on the single mutation investigated. Although so far, only a very limited subset of FALS-SOD1s has been investigated (Hayward et al., 2002; Rodriguez et al., 2002; Tiwari et al., 2003), experimental data acquired with recombinant proteins or *in vivo* implies that a certain degree of heterogeneity exists among mutations. Consequently, the mechanisms through which expression of the mutant SOD1 enzymes result in motoneuron injury and death remain controversial. It is believed, on the whole, that the gain of a novel toxic function by the SOD1 enzyme is responsible for the acquisition of the pathological phenotype. As to whether this function resides (1) in the tendency of mutant enzymes (as a consequence of either alteration of the β-barrel structure or disruption of the dimer interface) to form insoluble aggregates thereby obstructing intracellular functions, or (2) in a novel enzyme activity (as a consequence of alteration of the metal-binding active site), or (3) in disruption of metal homeostasis is presently debated.

As mentioned previously, SOD1 is an abundant component of many cell types, and accounts for up to 1% of total cytoplasmic proteins in some areas of the CNS (Pardo et al., 1995). It has been proposed that formation of insoluble aggregates of misfolded mutant SOD1 may play a part in FALS associated cell death, in a fashion similar to that which occurs in other neurodegenerative "conformational" diseases. As to whether SOD1 aggregates symbolize a cause, a correlate or a consequence of processes leading to cell death is still debated (Brown, 1998). Neurons and astrocytes of SALS patients are known to contain cytoplasmic aggregates that exhibit immunoreactivity for SOD1 and ubiquitin similar to inclusion bodies observed in SOD1-linked FALS patients (Bruijn et al., 1998). SOD1-associated FALS mutants have altered solubility and enhanced stability as a result of

inhibition of the degradation activity of proteasomes (Shinder et al., 2001). Recently, the relevance of SOD1 aggregates in FALS pathology has been questioned, with for instance, reports that the formation of SOD1 aggregates is independent of induction of cell death (Lee et al., 2002; Takeuchi et al., 2002).

The toxicity of SOD1 aggregates may arise either through the sequestration of other proteins required for neuronal function or through impairment of transport of other substrates vital for neuronal viability (Julien, 2001). In addition, SOD1 aggregates may reduce proteasome activity required for normal protein turnover (Urushitani et al., 2002). This may be critical, given that the proteasome not only plays a part in the degradation of misfolded proteins, but also in the regulation of transcription factors.

### 1.3.1 Clinical Differences of Different Mutations.

The most common FALS mutation, an alanine to valine shift at codon 4 (A4V), is coincidently the first within the SOD1 gene and accounts for 50% of all reported mutations in North America (Rowland, 1998). This mutation results in the most aggressive form of FALS, and exhibits an unusually short survival of only 1.5 years compared to the histidine to arginine shift at position 46 (H46R) which has an average life expectancy of 18 years following disease onset (Ratovitski et al., 1999).

A study carried out by Cudkowicz et al. (1997) of FALS-associated-SOD1 mutations established a number of findings. Firstly it was established that SOD1 mutations are specific to FALS. On the whole, FALS with or without SOD1 mutations are clinically similar but the age of onset in FALS with a SOD1 mutation is slightly earlier, with a mean age of 46.9 years compared to non-SOD1 patients where the mean age is 50.5 years (Cudkowicz et al., 1997). However, some clinical characteristics of SOD1 FALS cases depend on the specific mutation, for example, upper motor neuron signs are always absent in SOD1 A4V cases (Cudkowicz et al., 1998). Mutations are found in approximately 23% of families, highlighting the possible existence of at least one more disease gene.

There is a sizeable variation in disease severity as measured by age at symptom onset and disease duration. Of all the clinical variables tested, bulbar onset and two specific mutations were demonstrated to modulate age of onset or survival. Generally bulbar-onset patients are older when their illness begins whereas individuals with the G37R and L38V mutations display an earlier age of onset. In terms of survival, the A4V mutation is associated with shorter survival, compared to mutations G37R, G41D, and G93A, which predict longer disease duration. It is important to note that mutations that predict earlier disease onset such as G37R and L38V are not the same as those that are associated with shorter duration of disease, such as A4V, suggesting that the factors that modulate timing of disease differ from those implicated in rate of progression of the disease (Cudkowicz et al., 1997).

Age of onset varies with mutations, for instance the mutation L106V is associated with the earliest onset and has a mean age of 35.5 years and I113T has the latest onset with a mean age of 58.9 years (Cudkowicz et al., 1997). The fact that I113T was found to have the latest age of onset may in some way account for the finding of this mutation in many apparently sporadic ALS cases (Deng et al., 1995; Jones et al., 1993 & 1994). It is possible that relatives of these apparently sporadic cases died before reaching this late age of onset, as a result eliminating any family history. Nevertheless, other SOD1 mutations have also been identified in SALS (Orrell, 2000).

The first study of the structural location of FALS mutations within SOD1 revealed from the 12 mutations investigated that the mutations altered conserved interactions crucial to structural folding and dimer contact, rather than catalysis (Deng et al., 1993). Thus far, the majority of mutations have been found to affect the backbone of the protein or regions involved in dimerization of the two subunits. The position and type of amino acid modification may determine the potential effect on the protein and its function. The H46R and H48Q mutations are two interesting mutations that have been detected in highly conserved residues within the copper-binding domain. The H46R mutation has been described in Japanese ALS families and is associated with a slowly progressive phenotype and a relatively long survival of approximately 17.3 years (Aoki et al., 1993). In comparison, in a British ALS family the nearby residue 48 is mutated; this H48Q shift is

associated with a severe phenotype with rapid progression and a very short survival, of only 8 months (Enayat et al., 1995; Shaw et al., 1997). The markedly different effect of these two mutations accentuates the point that SOD1 mutations have quite varied effects on phenotype and prognosis (Enayat et al., 1995; Orrell et al., 1999).

### 1.3.2 Effect of SOD1 Mutations

The discovery of the association between SOD1 mutations and ALS led to a lot of speculation as to the mechanism by which the mutant SOD1 could trigger ALS. Initially, given the crucial role of SOD1 in detoxifying free radicals, it was attractive to propose that mutations in the enzyme resulted in reduced activity, thus permitting the cellular accumulation of harmful superoxide (Rosen et al., 1993). There have been reports of reduced SOD1 activity in the cerebrospinal fluid (CSF) of ALS patients (Bracco et al., 1991) and free radicals have been implicated in neuronal injury in several neurological disorders (Rosen et al., 1993). Alternatively, it was thought that overactive mutant SOD might produce excessive amounts of hydrogen peroxide, which may be rapidly converted, in the presence of ferrous ions, to the more toxic hydroxyl radical (Fullerton et al., 1998; Imlay et al., 1988). A dominant negative effect would mean that not only would the mutant be dysfunctional, but also additionally it could impair the activity of the normal enzyme, resulting in a reduction in activity of more than 50% (Deng et al., 1993). On the other hand, a mutation could cause the enzyme to gain a function. However, none of these hypotheses addressed how a ubiquitously expressed gene could result in such selective motor neuron death.

An initial approach undertaken to answer these questions was to determine the activity of the mutant SOD1. Analysis of red blood cell enzyme activity of six individuals with different SOD1 mutations, established average mutant activity to be less than half (41%) that of controls, providing support for the dominant negative theory (Deng et al., 1993). A study by Rosen et al. (1994) on the A4V SOD1 mutation, which results in a clinically severe FALS phenotype, found that in lymphoblastoid cell lines the SOD1 enzyme activity was 50% that of normal; protein levels were reduced compared to normals, but not

to 50%. In sporadic ALS cases and FALS cases without SOD1 mutations activities were normal (Garofalo et al., 1995).

Not all FALS mutations bring about a decrease in SOD1 activity, as demonstrated by the study of the G37R mutation, which retains full specific activity, but displays a two-fold reduction in polypeptide stability, resulting in an overall reduction to 40-60% of normal enzyme activity. The G85R SOD1 mutation, on the other hand, results in the production of an inactive protein. In order to assess the influence of the mutant on the stability or activity of the normal enzyme, SOD1 mutants with reduced half-life and reduced free radical scavenging abilities were expressed with normal SOD1. Normal subunits were found to be unaffected in activity or half-life (Borchelt et al., 1995). In general, enzymes are present in excess amounts. Therefore one cannot help but wonder how, especially in the case of SOD where three isoforms of the enzyme are found to be present, such a slight reduction is sufficient to produce disease. Loss of function mutations commonly produce autosomal recessive phenotypes, and even in heterozygous carriers of recessive mutation where 50% reduction of enzyme is found, individuals are clinically unaffected. As a result of the difficulty in reproducing erythrocyte and CSF enzyme activity results (Rowland, 1995), transgenic mice expressing wild type and mutant forms of human SOD1 have been developed as models for ALS.

Mice expressing mutant G93A-SOD1 developed motor neuron disease that was not observed in mice expressing wild-type SOD1 at similar levels (Gurney et al., 1994). In mice, expression of G86R-SOD1 mutation, which corresponds to human G85R, resulted in motor neuron degeneration, even though there was no reduction in activity of SOD (Ripps et al., 1995). It was shown by Wong and colleagues (Wong et al., 1995) that although transgenic mice expressing human G37R-SOD1 develop severe motor neuron disease, mice expressing the wild-type human SOD1 gene at similar and higher levels do not exhibit the disease state. In addition, another important study created mice completely deficient for the SOD1 enzyme by deletion of the entire coding sequence of mouse SOD1 gene (Reaume et al., 1996). These animals develop normally and exhibit no signs of motor neuron disease; however they exhibit increased vulnerability to motor neuron loss following axonal injury, suggesting that SOD1 may play a larger role in situations of injury as opposed to development and function (Siddique et al., 1996). This suggests that motor neuron death comes about as a result of

mutant-mediated toxicity as shown by the development of disease in transgenic mice with mutant SOD1 in the absence or elevation of wild-type SOD1 (Bruijn et al., 1998). Yeast mutants null for SOD1 enzyme were rescued just as efficiently by FALS-associated SOD1 mutants as by wild type, substantiating the activity of the mutant enzyme (Rabizadeh et al., 1995).

In neuronal cells, over-expression of wild-type SOD1 inhibits apoptosis whereas over-expression of FALS-associated SOD1-mutants promotes apoptosis, supporting a gain-of-function effect (Rabizadeh et al., 1995). Additionally, it was observed that no SOD1 null mutations had been found in FALS patients as would be the case if loss of activity had been the cause of the disease (Brown, 1995). All this observed and experimental evidence gave support to the concept that FALS is caused not as a result of loss or reduction in SOD1 activity, but rather by the acquisition of a harmful toxic property by the mutant enzyme.

### 1.3.3 Proposed Toxicity of Mutant SOD1

Three of the properties of mutant SOD1 that have been investigated include altered substrate reactivity, metal toxicity, and abnormal interactions with proteins. Altered reactivity for certain substrates has been observed for mutant SOD1 enzyme. Dismutase activity of FALS-associated SOD1 mutants is normal but peroxidase activity is enhanced (Wiedau-Pazos et al., 1996; Yim et al., 1996). The peroxidase reaction is copper-dependent as the mutant reaction is more sensitive to inhibition by copper chelators than the wild-type enzymes. X-ray crystallographic studies have revealed the active channel of FALS mutants to be slightly larger than the wild type, providing support to the theory that $H_2O_2$ now has access to the mutant enzyme (Yim et al., 1996). The subsequently generated hydroxyl radical has a number of possible fates, either it may react with a histidine residue within the active site, thus inactivating the enzyme or it could react with molecules within the active site, or simply exit the active site and react with other molecules (Bredesen et al., 1996).

Following the discovery of FALS mutations in the SOD1 gene, it was thought that as a dominant disorder, mutations would bring about at most a 50% reduction in SOD1 activity, thus doubling the steady-state concentration of superoxide within the cell.

Superoxide reacts with nitric oxide in a reaction that is three times faster than that with wild-type SOD1 resulting in the formation of peroxynitrite, which in turn can then form nitronium-like intermediates capable of nitrating tyrosine residues (Beckman et al., 1993). Increased nitrotyrosine levels have been reported in sporadic and familial ALS cases as well as in animals (Abe et al., 1995; Beal et al., 1997; Ferrante et al., 1997).

It has been shown that mutant SOD1 has reduced stability (Alexianu et al., 1998) which could mean that there may be reduced buffering of copper and zinc, both of which can be neurotoxic (Brown, 1995). The H46R and H48Q SOD1 mutations directly affect copper-binding ligands and consequently these mutants cannot bind any copper (Casareno et al., 1998; Siddique et al., 1996). The zinc-binding site of the enzyme can be dramatically altered by FALS mutants, consequently enabling the mutant to influence the redox behaviour of the enzyme (Lyons et al., 1996). Interestingly, it is thought that mutations throughout the entire protein may have this same effect on the zinc-binding site thereby accounting for the fact that so many different mutations can yield a common toxic property. The affinity for zinc in the mutant enzyme is thought to be reduced by up to 30-fold in comparison to wild-type (Crow et al., 1997). Nevertheless, measurements of concentrations of total copper and zinc in both sporadic and familial ALS cases with a range of controls did not uncover any relationship between free Cu and Zn with disease, severity, age of disease onset, or SOD1 activity (Cha et al., 2000).

The instability of the mutated protein may be sufficient for the mutant protein to precipitate and form toxic aggregates (Brown, 1995). This is important considering that SOD1 represents approximately 1% of cytosolic protein therefore changes in folding, solubility, or degradation of such an abundant protein may potentially result in aggregates. This is consistent with the detection of SOD1 immunoreactive inclusion bodies in motor neurons expressing mutant SOD1 but not with wild-type SOD1 (Bredesen et al., 1996; Bruijn et al., 1998; Durham et al., 1997). A recent study in a mutant SOD1 mouse model of ALS has shown aggregation and formation of inclusion bodies, to be distinct processes (Bracco et al., 1991). In addition, aggregation is seen as an early event in disease pathogenesis and may be used as a biochemical marker, whereas inclusion-body formation

takes place later via microtubule retrograde transport and is detectable only after manifestation of the disease.

Mutant SOD1 displays at least two novel protein interactions, which can be detected by a yeast interaction trap system (Kunst et al., 1997). One is with Lysyl-tRNA synthase (KARS), which is involved in protein synthesis and the other with translocon-associated protein delta (TRAP-δ), which is involved in transport of newly synthesized proteins into the endoplasmic reticulum. It is believed that interaction between mutant SOD1 and either of these proteins could result in abnormal localization or function and as a result prevent the transfer of important proteins essential to cell survival or even lead to formation of aggregates; this may divert the actions of chaperones from other important processes and lead to dire consequences.

## 1.4 Mechanisms of motor neuron degeneration in ALS

Several mechanisms for the degeneration of motor neurons in ALS have been put forward. In this section I will discuss the mechanisms most relevant to familial ALS, which are oxidative stress, copper toxicity, neurofilaments, glutamate excitotoxicity, apoptosis, and the basis of selectivity of cell death.

### 1.4.1 Oxidative Stress

Oxidative stress is the condition which arises as a result of an imbalance between the physiological production of potentially toxic reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl radical which are products of normal metabolism of molecular oxygen (Coyle et al., 1993) and the physiological scavenging activities. These include enzymatic activities (superoxide dismutase, catalase, peroxidase and peroxiredoxin), low molecular weight antioxidant species (Vitamin E, ascorbate, glutathione), in addition to more complex forms of protection such as systems for transport and buffering of metals and induction of transcription factors. Intracellular antioxidant defence can be thought of as acting at three levels: prevention of damage, i.e. prevention of

electron leakage which results in generation of ROS; interception of potentially dangerous ROS, i.e. scavenging by antioxidant molecules; and repair of damage, i.e. removal of molecules which have been damaged by ROS (Almer et al., 2001 & 2002). In this process, the proper handling of transition metal ions such as copper and iron is essential, as these metals are able to undergo redox cycling and as a result generate toxic ROS.

Different tissues differ in their susceptibility to oxidative stress. The central nervous system (CNS) is particularly sensitive to this type of damage for several reasons such as low levels of a number of antioxidant enzymes (catalase and GSH-peroxidase); a high content of easily oxidised substrates (e.g. membrane polyunsaturated lipids); and an inherently high flux of ROS generated through neurochemical reactions such as dopamine oxidation (Halliwell, 1992). Oxidative stress in nervous tissue can be detrimental as a result of several interacting mechanisms, including direct damage to vital molecular species, increase in free intracellular $Ca^{2+}$ and release of excitatory amino acids.

A role for ROS-mediated oxidative stress in ALS, was put forward by a number of investigators who reported elevation of typical oxidation products such as malondialdehyde, hydroxynonenal, oxidised proteins, DNA and membrane phospholipids in both sporadic and familial ALS patients, as well as in several model systems (Andrus et al., 1998; Beal et al., 1997; Bogdanov et al., 2000; Bogdanov et al., 1998; Ferrante et al., 1997; Fitzmaurice et al., 1996; Gurney et al., 1994; Hall et al., 1998; Liu et al., 1998; Liu et al., 1999; Rizzardini et al., 2003; Shaw et al., 1995; Shibata et al., 2001).

Damage brought about by free radicals may become prominent in mitochondria, where the majority of the cellular production of ROS occurs and whose harmful effects are directed toward mitochondrial proteins, lipids, as well as mitochondrial DNA. Almost all of the energy of the brain is derived from oxidative metabolism of the mitochondrial respiratory chain. Brain mitochondria are particularly vulnerable to oxidative damage as a result of the extensive generation of cellular oxidants by this organ. There exists a considerable amount of evidence of mitochondria damage in both SALS and FALS patients (Beal, 2000; Borthwick et al., 1999; Brown, 1998; Curti et al., 1996; Dhaliwal et al., 2000; Fujita et al., 1996; Nakano et al., 1987; Swerdlow et al., 1998) that can be ascribed to

intracellular oxidative stress. Such damage may lead to the triggering of apoptotic cell death of motor neurones, thought to take place in ALS (Guegan et al., 2001; Martin, 1999). ROS are extremely well known inducers of cell death and are known to regulate both early and late stages of apoptosis; protection against apoptosis can be achieved through inhibition of ROS production (Fleury et al., 2002).

Reactive oxygen species (ROS) have also been implicated in aging and overexpression of SOD1, and SOD/catalase mimetics have been found to dramatically extend lifespan in both Drosophila (Parkes et al., 1998) and *C. elegans* (Melov et al., 2000). Evidence suggests an age-related accumulation in oxidative damage to DNA, and in particular mitochondrial DNA, in the brain (Mecocci et al., 1993). The oxidative stress theory provides an attractive explanation for ALS, because it accounts for cumulative damage in a late-onset, progressive disease as well as places the major target for damage as the central nervous system. The argument for oxidative stress as a mechanism of disease is strengthened by the finding of mutations in the Cu, Zn superoxide dismutase (SOD1) gene in a number of FALS cases (Deng et al., 1993; Rosen et al., 1993). A reduction in SOD1 activity does not cause ALS, some SOD1 FALS mutants have reduced free radical scavenging ability (Borchelt et al., 1995), and chronic inhibition of SOD1 activity leads to motor neuron degeneration, reversed by treatment with antioxidants (Rothstein et al., 1994).

The spontaneous reaction of superoxide with nitric oxide leads to the formation of peroxynitrite and the subsequent formation of highly reactive nitronium intermediates capable of nitrating tyrosine groups of proteins and forming the stable 3-nitrotyrosine compound (Beckman et al., 1993; Cha et al., 2000). It is thought that nitric oxide is not present in motor neurons, but NOS is induced following injury (Bruijn et al., 1997). Increased levels of nitrotyrosine have been reported in sporadic and familial ALS cases as well as in animals (Abe et al., 1995; Beal et al., 1997; Ferrante et al., 1997; Tohgi et al., 1999). *In vivo* evidence of increased tyrosine nitration, which is coincident with the earliest pathological abnormalities, comes from studies of 3-nitrotyrosine levels in SOD1 G37R mouse. Nonetheless, no increase in protein-bound nitrotyrosine was detected at any stage (Bruijn et al., 1997).

There is other evidence of oxidative pathology in the disease besides elevated levels of nitrosylated proteins such as an increase in damage to protein (Shaw et al., 1995), DNA, and phospholipids (Bogdanov et al., 2000; Ferrante et al., 1997; Smith et al., 1998). Furthermore, there is an increase in metallothionein immunoreactivity in ALS (Sillevis Smitt et al., 1992). Metallothionein functions in metal homeostasis (in particular $Zn^{2+}$) and detoxification, as well as in free radical scavenging. The increases in ALS are believed to be local, possibly as a result of slower breakdown (Blaauwgeers et al., 1996; Radunovic et al., 1997).

On the other hand, the ability of SOD1 mutants to utilise $H_2O_2$ as a substrate, probably as a result of increased availability of the $Cu^{2+}$ site, may result in generation of hydroxyl radicals and cellular damage. The most reactive species, which is the hydroxyl radical, is not generated directly by any known enzymatic reaction, but $H_2O_2$ slowly decomposes to $^{\cdot}OH$, a reaction that is accelerated in the presence of $Fe^{2+}$ (Coyle et al., 1993). A number of SOD1 mutations have exhibited enhanced copper-dependent peroxidase activity (Bogdanov et al., 1998; Liu et al., 1998; Wiedau-Pazos et al., 1996; Yim et al., 1996), although this finding has been disputed by some reports (Singh et al., 1998). Alterations in the structure of SOD1 mutants suggest that $H_2O_2$ has access to the mutant enzyme (Yim et al., 1996). ALS associated mutant SOD1 has reduced zinc affinity compared to NF subunits which possess a high affinity that is sufficient enough to remove zinc from SOD. This may as a result lead to a reduction in superoxide scavenging and increased catalysis of tyrosine nitration by peroxynitrite (Crow et al., 1997).

Other lines of evidence suggest that novel properties of the FALS-associated SOD1 mutant enzyme involve imbalance of ROS. It remains unclear as to how mutant SOD1 causes oxidative stress, and which molecules represent direct targets/propagators of damage, however evidence is accumulating in relation to the role of SOD1-bound copper ions in mediating oxidative damage toward other proteins, for instance those involved in mechanisms of intracellular signal transduction (Casciati et al., 2002; Ferri et al., 2001; Volkel et al., 2001).

FALS-associated SOD1 mutants may elicit an imbalance of ROS metabolism as a result of their imperfect folding, possibly leading to a loosening of protein structure; this would in turn result in the modification of the active site and alteration in the substrate specificity of the enzyme. For instance, as suggested by studies *in vitro* (Wiedau-Pazos et al., 1996) and *in vivo* in yeast (Roe et al., 2002), modification of copper geometry at the active site could exacerbate the reported peroxidative activity of SOD1.

It is not clear as to whether elevated oxidative stress and protein aggregation are causally connected. In addition, it is not known whether FALS-associated mutant SOD1 proteins found in aggregates are modified (cleaved, oxidised, de-metallated or otherwise covalently modified) (Valentine, 2002). It is possible that aggregation of protein is caused by the increased oxidative reactivity of misfolded mutant SOD1 or that the aggregates themselves are the mediators of the oxidative reactions. AGE-modified FALS-associated SOD1 has been detected both in neuronal Lewy body-like hyaline inclusions as well as astrocytic hyaline inclusions of FALS patients and transgenic mice (Valentine, 2002). Additionally, it has been suggested that mutant SOD1 that is aggregated, but still active, may mediate the formation of ROS. It has also been proposed that misfolded SOD1 may contribute to generation of intracellular ROS by loss of the ability to buffer copper.

There are several defence mechanisms committed to reducing the levels of oxidants such as SOD, catalase, ascorbic acid (vitamin C), and α-tocopherol (vitamin E). An imbalance between oxidant and antioxidant mechanisms may result in neuronal damage, as observed in vitamin E deficiency, which may lead to neurological symptoms (Coyle et al., 1993). In ALS, the benefit of antioxidants was demonstrated by vitamin E treatment of transgenic SOD1 mutant mice (Gurney et al., 1996) as well as in cell cultures (Troy et al., 1994). Clinical trials of antioxidants however, have been rare and not as successful as expected. No increased survival was observed in ALS patients on treatment with a selection of free radical scavenging antioxidants (Louwerse et al., 1995; Vyth et al., 1996). Given the evidence of oxidative damage, these conflicting data could be the result of incorrect dosing, inappropriate patient selection, or choice of antioxidant for the particular disease under consideration (Delanty et al., 2000). Furthermore, it is very likely that the complex biochemistry of free radicals may not lend itself to such treatments.

### 1.4.2 Copper Toxicity

Redox-active transition metals are useful but dangerous trace elements. Copper is essential to the life of cells and acts as a cofactor for many enzymes, however, if copper ions are not properly transported, stored or utilised, redox reactivity results in risk of damage to cells and tissues.

Under normal conditions, intracellular "free" copper ions are virtually non existent (Rae et al., 1999) as a complex mechanism of copper buffering has evolved to prevent metal-mediated oxidative stress. Copper buffering is achieved through the simultaneous action of a number of proteins in a process than can be thought of as a chain of consequent events regulating extracellular transport, uptake, intracellular delivery from chaperones (e.g. copper chaperone for SOD1 (CCS), COX17 and Atx1) to specific targets (such as SOD1 and cytochrome c oxidase) and/or storage in copper scavenging systems (such as GSH and metallothioneins) (Puig et al., 2002).

If intracellular uptake is impaired, inactivation of copper-dependent proteins takes place with loss of their function. This is not the case in SOD1-associated FALS, in which no change is observed in total copper content, not even in experimental conditions in which CCS is missing (Subramaniam et al., 2002).

Once copper ions are inside the cell they must be dealt with appropriately, by chaperoning them to substrates, either enzymes or thioneines. When these substrates are unavailable, absent, or unable to effectively bind the copper, the cell is presented with an unfavourable situation in which there is an excess amount of toxic, radical-generating metal ions. This may be taking place in SOD1-associated FALS, as it is known that several SOD1 mutants no longer possess the ability to bind metals properly *in vitro* and possibly *in vivo* (Hayward et al., 2002). Although differences among different SOD1 mutants in their ability to bind copper (and zinc) have been reported (Hayward et al., 2002), this appears to be to a general characteristic of FALS-associated SOD1, supporting the previously suggested hypothesis that SOD1 plays a key role in the buffering of copper. This hypothesis is based on the observation, of the existence of a pool of inactive copper-free enzyme, in human cell

models; this inactive enzyme is re-activated in response to copper overload and may symbolize a secondary defence to metal-induced oxidative stress (Petrovic et al., 1996; Steinkuhler et al., 1991 & 1994).

In man, SOD1 is present in a single copy per haploid genome and FALS is a dominantly inherited disease which has a penetrance close to 1, with patients being in general heterozygous for mutant SOD1. Therefore, alteration of half of SOD1 molecules in patients may lead to an imbalance of either copper buffering or copper chemistry, when bearing in mind that SOD1 is a very abundant protein, representing up to 1% of total proteins in several cell types. The notion of "aberrant copper chemistry" of FALS-associated SOD1 was initially suggested in pioneering studies on altered geometry of the active site (Carrì et al., 1994) and reactivity of mutant proteins *in vitro* (Wiedau-Pazos et al., 1996; Yim et al., 1996). Subsequent studies in cells expressing FALS-associated mutant SOD1, supported a role for alteration of copper as a reason for increased oxidative stress (Corson et al., 1998; Gabbianelli et al., 1999).

In the SOD1 dimer, each subunit contains one atom of both $Cu^{2+}$ and $Zn^{2+}$ (Siddique et al., 1996). Since copper may be neurotoxic, one possible path of investigation was the affinity with which the mutant SOD1 molecules might bind the copper atom. Conformational alterations in the molecule such as relaxed folding may lead to exposure of the copper atom or permit it to interact with other molecules. The observation that copper chelators prevent the abnormal effect of FALS-associated-SOD1-mutants provides support to the importance of copper (Azzouz et al., 2000; Gabbianelli et al., 1999; Ghadge et al., 1997; Wiedau-Pazos et al., 1996). Investigation of the D90A SOD1 mutation revealed normal binding of copper (Marklund et al., 1997). An investigation of a number of SOD1 mutants identified a common property, the ability to bind copper, which is accomplished by means of the copper chaperone for SOD (CCS). This proposed a mechanism for SOD1 mutant-mediated disease as being through aberrant copper chemistry as well as a possible therapeutic approach through the use of CCS inhibitors (Corson et al., 1998).

Copper is specifically delivered to SOD1 by CCS, and yeast cells with a null mutation in the yeast equivalent (LYS7) have normal levels of SOD1 however they fail to

incorporate copper and as a result have no superoxide scavenging activity (Culotta et al., 1997). There is strong homology between CCS and SOD1 molecules with conservation of the amino acids essential for mediating SOD1 homodimerization and it is through this region that the interaction between CCS both with wild-type SOD1 and FALS-SOD1-mutants takes place (Casareno et al., 1998). More importantly, this region of homology also contains most of the copper and zinc ligands, in addition to most of the amino acids mutated in FALS (Casareno et al., 1998). The H46R and H48Q SOD1 mutations contain no copper because of the absence of an essential ligand, however both interact with CCS. It was proposed that the chaperone interacts with these mutants and attempts to deliver copper, even if the enzyme is not able to accept it, resulting in the release of copper into the cell and subsequent toxicity (Casareno et al., 1998).

This model is consistent with the absence of an ALS phenotype in SOD1 null mice, as without an interaction between SOD1 and CCS, transfer of copper cannot take place. This theory is supported by mice with targeted disruption of the CCS gene, which display a similar phenotype to SOD1 null mice (Wong et al., 2000). Although these mice are viable and express normal levels of SOD1 protein, they have reduced SOD1 activity. This decrease in activity is the result of a defect in the incorporation of copper into SOD1, and other copper manipulations for instance uptake, distribution, or incorporation into other enzymes remain unchanged (Wong et al., 2000). The free intracellular copper concentration is very low, which implies that the copper chelating activity of the cell is high and that the CCS is required to deliver copper to SOD1 (Rae et al., 1999). In yeast the absence of CCS does not change the number of SOD1 molecules, however it does reduce the ratio of active, copper-containing enzymes to inactive, copper-less molecules (apo form). Further support for a role of CCS in the disease mechanism in ALS comes from the co-localization of CCS and SOD1 within degenerating cells (Casareno et al., 1998; Rothstein et al., 1999). Potential therapeutic approaches may comprise CCS inhibition (Corson et al., 1998) or disruption of the interaction of CCS-SOD1 to prevent copper-mediated toxicity (Casareno et al., 1998). The only reported study in two ALS pedigrees of the CCS gene on chromosome 11 was unsuccessful in detecting linkage (Orlacchio et al., 2000).

Further support to the notion of altered metal homeostasis in ALS comes from the observation of increased levels of expression of metallothioneins (MT), in the spinal cords of patients (Sillevis-Smitt et al., 1992) and FALS transgenic mice (Gong et al., 2000), where changes in MT expression take place prior to the onset of motor deficits or significant motor neuron pathology. In addition, FALS mice arrive at the onset of clinical signs and death considerably earlier in response to the reduction of MT expression (Nagano et al., 2001; Puttaparthi et al., 2002). These findings are consistent with the hypothesis that the metal-mediated free radical production brought about by either mutant SOD1 or mishandled metal ions might be associated with the degeneration of motor neurons in FALS.

Lastly, a possible relationship between ALS and metal homeostasis is represented by modulation of proteasome functionality. It has been suggested that overexpression of some FALS-SOD1-mutants such as A4V, G85A or G93A, may result in neuronal death through inhibition of proteasome activity, as a result of accumulation of mutated/misfolded protein with time (Urushitani et al., 2002). One may speculate that, upon moderate oxidative stress, cell proteolytic activity is increased as a "scavenging" response with the intention of removal of oxidised products; this initial step may or may not be accompanied by formation of small aggregates, depending on the cell type or on the extent of production of ROS. When the damage from the oxidative stress exceeds a critical threshold, the activity of the proteasome is impaired as a result of it being over loaded. Reduction of proteasome activity required for normal turnover of protein would result in malfunctioning of overall cell metabolism.

## 1.5 Susceptibility Genes

Taking into consideration that mutations in ALS genes occur in patients with familial as well as sporadic ALS, it is likely that more than 10% of ALS can be explained by the multiple autosomal-dominant and –recessive forms. Since at present, there is no evidence for any specific environmental cause for sporadic or familial ALS, most remaining cases of the disease are thought to be triggered by the interaction of several genes and environmental factors.

I will now describe here some of the genes potentially contributing to the development of ALS. For the context of this thesis, these genes are referred to as "susceptibility genes", as mutations in these genes may only lead to ALS in the presence of other genetic or environmental risk factors.

### 1.5.1 Neurofilaments

One of the most commonly reported pathological findings in ALS is the aberrant accumulation of neurofilaments in perikarya and proximal axons of motor neurons, however it remains unclear as to whether this has a central role in the disease process or is merely a harmless by-product (Cleveland et al., 1996). The neurofilaments are a major component of the axonal cytoskeleton and since motor neurons possess very large NF-rich axons, this structural attribute is one of the strongest clues to the selective vulnerability of motor neurons in ALS. A role of neurofilaments is to maintain axon structure and calibre (Julien et al., 1999). In ALS, axonal loss is size-dependent, with principal loss being of large fibers (Sobue et al., 1987).

The first experimental evidence for the involvement of neurofilaments in the pathogenesis of ALS came from the generation of mouse models expressing high levels (three to four time's normal) of wild-type mouse NF-L (Xu et al., 1993) and wild-type human NF-H (Cote et al., 1993). In these animals, NF accumulation was associated with axonal degeneration, proximal swelling, and severe skeletal muscle atrophy, suggesting that neurodegeneration can be triggered by the accumulation of NF in motor neurons. There was however, no significant loss of motor neurons and in addition the extent of overexpression caused some trepidation about the specificity of the toxic effect as well as the rate of protein degradation. Consequently, a mouse model was created which expressed only a modest level of mutant NF-L, and this gave rise to selective degeneration of motor neurons accompanied by NF accumulation (Lee et al., 1994). Further support for the role of NF in the disease came from the description of severe neurofilamentous pathology in association with an SOD1 mutation in an ALS individual (Rouleau et al., 1996).

Although NFs are obligate heteropolymers, each of the three NF subunits can form heterodimers with peripherin which is another neuronal intermediate filament (IF) protein and is also a component of the IF inclusion bodies seen in ALS. The expression of peripherin with NF-H and NF-M, in the absence of NF-L, prevents IF formation in cells (Beaulieu et al., 1999). Further studies investigating the role of peripherin in ALS show that sustained overexpression of wild-type peripherin in mice triggers massive and selective degeneration of motor axons during ageing. Onset of peripherin-mediated disease was brought about by a deficiency of NF-L protein (Beaulieu et al., 1999). In mice, the overexpression of NF-L can rescue the NF-H phenotype (Meier et al., 1999). However, in mice null for NF-L and overexpressing NF-H, protein aggregates occur in the absence of motor neuron death, whereas animals with overexpression of peripherin have toxic inclusions, which result in death (Beaulieu et al., 2000). These findings suggest that the type and location of the IF aggregates may play a part in its toxicity.

Mice carrying the G85R SOD1 mutation and a deleted NF-L gene display a slower disease onset and reduced disease progression (Williamson et al., 1998); indicating neuronal vulnerability to SOD1 mutant toxicity is reduced in the presence of lower levels of NF. However, overexpression of human NF-H in G37R-SOD1 mutant mice results in a 65% increase in lifespan of the G37R animals (Couillard-Després et al., 1998). Confirmation of these findings comes from a study in which both NF-L and NF-H were overexpressed with another SOD1 mutant (Kong et al., 2000). In mice, the overexpression of human NF-L with G37R-mutant-SOD1, leads to excess NF in motor axons but does not accelerate the disease process mediated by the mutant SOD1 enzyme, suggesting that the increase of NF may protect motor neurons from SOD1 mediated toxicity (Couillard-Després et al., 2000). One mechanism that can explain this finding is that extra NF proteins may act as a sink for toxic oxygen radicals, however no differences in pattern or amount of nitrotyrosine or carbonyl modifications have been observed, suggesting that nitration occurs normally within neurons (Strong, 1999). In sporadic ALS cases selective suppression of NF-L mRNA has been observed without any alteration to mRNA levels of other NF subunits or neuronal nitric oxide synthase (NOS); since NF-L is thought to function as a sink for free reactive nitrating species this suggests that the motor neurons are unable to effectively buffer reactive nitrating species (Strong, 1999).

An alternative hypothesis for the way in which neurofilaments play a part in the mechanism and selectivity of motor neuron disease comes from the neurofilament-dependent slowing of axonal transport as neurofilamentous accumulation occurs (Williamson et al., 1999). The first evidence that mutations in SOD1 modified the NF network came from an SOD1 G93A mouse model in which axonal transport was impaired (Zhang et al., 1997). Proteins synthesized in the neuron cell body are delivered by means of axonal transport to the axon, which has both fast and slow components. The slow transport component may be subdivided again, in accordance with the speed of the movement, into slow component a (SCa ~0.5 mm $day^{-1}$) which transports NF proteins, tubulin, actin, and slow component b (SCb ~1-2 mm $day^{-1}$), which also carries tubulin and actin along with other proteins (Williamson et al., 1999). In transgenic mice expressing human SOD1 -G37R or -G85R, examination of rate and composition of slow axonal transport revealed that reduced slow transport was evident months ahead of clinical features of disease (Williamson et al., 1999). In the G37R mice, there was a marked reduction in the transport of all three subunits of NF, by a factor of two, whereas fast transport remains unaffected.

In addition, it was found that by limiting the expression of NF within cells and by preventing the localization of NF in the axon, SOD1 mutant-mediated diseases in mice with or without axonal neurofilaments were impossible to differentiate; suggesting that axonal NF is not a prerequisite for disease pathology (Eyer et al., 1998). This indicates that the disruptions of NF observed in ALS are secondary. In a recent study NF content was reduced by disruption of each of the three NF genes and the ensuing effect was studied in the context of a SOD1 FALS mutation. The large decrease in NF caused a significant reduction in axonal calibre but did not prevent loss of motor neurons (Nguyen et al., 2000). The conclusions drawn from the study were that neither high NF burden nor large calibre axons could provide an explanation for the selective degeneration of motor neurons in ALS.

In summary, even though the accumulation of neurofilaments is a common pathological characteristic of ALS, the content of NF does not appear to be responsible for the selective death of motor neurons. The creation of several animal models with NF transgenes has provided insight into the role played by these elements in ALS. Recent evidence has highlighted both the interactions of various filament proteins and the

expression levels of each type of protein as having a strong influence on the course of the disease. In addition, it is becoming clear that the cellular location of NF accumulation, i.e. perikaryal versus axonal, may be crucial to the disruption of cellular function, in particular the axonal transport system. In sporadic ALS cases, mutations in the heavy NF subunit may be a risk factor, however they are not common.

### 1.5.2 Excitotoxicity Genes

The term excitotoxicity denotes a phenomenon in which the excessive or prolonged activation of excitatory amino acid receptors leads to damage and eventually death of the neurons involved. The activation of these receptors is normally transitory and results in depolarization and neuronal excitation. In the brain, glutamate is the principal excitatory neurotransmitter. It is released by a $Ca^{2+}$-dependent mechanism from vesicles in the presynaptic terminals and subsequently diffuses across the synaptic cleft where it activates specific receptors.

There are two types of glutamate receptors ionotropic and metabotropic. There are three families of ionotropic receptors: kainic acid (KA), N-methyl-D-aspartate (NMDA), and α-amino-3-hydroxy-5-methyl-4isoxazolepropionic acid (AMPA). There are also three groups of metabotropic glutamate receptors (mGluRs) of which a total of eight (1-8) subunits have been identified (Meldrum, 2000). Since elevated levels of glutamate may be neurotoxic, it is essential that there is rapid clearance of the glutamate from the synaptic and extracellular environment; this is the role of specific glutamate transporters in the surrounding astrocytes and neurons. A cascade of intracellular events such as calcium and sodium influx is triggered by the stimulation of the glutamate receptors and results in activation of oxidant generating pathways. It is thought that elevated activity may be responsible for neurotoxic effects (Rothstein, 1996). Activation of the ion-gated receptors leads to an influx of $Ca^{2+}$ into the cell, which exerts many changes, including cell swelling (Leigh et al., 1996) and results in the activation of a number of enzymes such as NOS which may lead to the formation of NO. In the presence of superoxide, the generated NO can react to produce harmful peroxynitrite, which results in nitrosylation and inactivation of several proteins.

Reduced levels of glutamate and aspartate in brain and spinal cord of patients provided the first sign of a possible role for glutamate excitotoxicity in ALS (Plaitakis et al., 1988). A dramatic increase in glutamate and aspartate levels in ALS were identified by CSF studies (Rothstein et al., 1990) however not all studies stand in agreement (Perry et al., 1990). Abnormal transport or metabolism was suggested as the underlying problem, as a result of the findings of ratios of the amino acids and products of metabolism (Rothstein et al., 1990). Additionally, as the same protein regulates the clearance of extracellular glutamate and aspartate in ALS and levels of both amino acids are altered, this would imply a defective or inefficient transport mechanism (Rothstein, 1996). In ALS patients, a defect in high-affinity glutamate transport with disease, region, and chemical specificity has been identified (Rothstein et al., 1992). Chronic inhibition of glutamate transport gives rise to slow neurotoxicity in spinal cord cultures, providing further support for defective glutamate transport in ALS (Rothstein et al., 1993). Neuronal cell death, which arises as a result of chronic inhibition of SOD1, is enhanced by blockage of glutamate transport and prevented by treatment with a glutamate receptor antagonist (Rothstein et al., 1994).

The identification of three glutamate transporters – EAAC1 (excitatory amino acid carrier-1), GLT-1 (rat glial glutamate transporter), and GLAST (rat glial glutamate and aspartate transporter) has enabled the specificity and localization of these transport proteins as well as the role of each subtype in ALS to be studied. EAAC1 is specific to neurons, while GLT-1 and GLAST are specific to astrocytes (Rothstein et al., 1995). Through use of anti-oligopeptide antibodies, it was established that GLT-1 is significantly reduced in ALS, whereas EAAC1 is modestly decreased and levels of GLAST remain unchanged (Rothstein et al., 1995). Importantly, the reduction in GLT-1 was found in some, but not all, ALS specimens, which may account for the lack of alterations in concentrations of amino acids in some samples (Perry et al., 1990). The levels of mRNA for each of these transporters were found to be unchanged which suggests the reduction in GLT-1 protein might be the result of a translational or posttranslational alteration (Bristol et al., 1996). Inhibition of synthesis of GLT-1 or GLAST, through use of antisense oligonucleotides, resulted in increased extracellular glutamate, excitatory neurodegeneration, and paralysis. Loss of neuronal EAAC1 did not result in a reduction in extracellular glutamate but produced mild toxicity

(Rothstein et al., 1996). This highlights glial transporters as the principal glutamate transporters and as being essential in preventing neurotoxicity.

Abnormal GLT-1 (also known as EAAT2, excitatory amino acid transporter 2) mRNA was identified in non-SOD1 familial as well as sporadic ALS cases (Lin et al., 1996), providing a possible explanation for the reduction in functional protein. About 60-70% of sporadic ALS patients exhibit a 30-95% loss of the EAAT2 protein in motor cortex and spinal cord (Lin et al., 1998). In ALS patients, several abnormal EAAT2 mRNAs have been identified in abundance in affected areas but not in other areas of the brain. These aberrant mRNAs are found to be present in approximately 65% of sporadic cases. Initial investigations suggest the proteins synthesized from these abnormal mRNAs are prone to rapid degradation and exert a dominant negative effect on the normal EAAT2 protein, leading to a loss of activity (Lin et al., 1998). Alternative splice forms of EAAT2 mRNA, which are non-pathogenic, have also been identified (Nagai et al., 1998).

There are several possible causes, which could account for the generation of this abnormal mRNA. The identification of multiple aberrant mRNAs suggests a defect in mRNA processing or alternatively, there may be mutations in the EAAT2 gene (Bai et al., 1998; Lin et al., 1998). To elucidate this both linkage and mutation analysis was used to study the EAAT2 gene on chromosome 11p. No linkage was detected in 16 ALS pedigrees however mutation screening identified one novel mutation in a SALS patient and two novel mutations in two siblings affected with FALS (Aoki et al., 1998). The SALS mutation may be functionally significant, but those that are present in the FALS cases do not modify the amino acid. It has been concluded that mutations in the gene for EAAT2 are rare and do not account for the frequent detection of abnormal mRNA. A subsequent study identified three novel EAAT2 transcripts, none of which were found to be disease-specific; indicating that abnormally spliced RNA is unlikely play a major role in the pathogenesis of ALS (Meyer et al., 1999). Recently, EAAT2 splice variants were detected not only in ALS patients but also in controls and among individuals with Alzheimer's disease, which suggests that qualitatively the presence of the variants may not explain ALS pathology, but that there may be some quantitative modifications that affect motor neuron degeneration (Honig et al., 2000).

Currently, riluzole is the only effective approved treatment for ALS, which has a neuroprotective role, which may be due to its ability to inhibit presynaptic glutamate release (Doble, 1996). Treatment with riluzole has been shown to prolong survival in both ALS patients and in animal models (Gurney et al., 1996; Lacomblez et al., 1996). Extended survival has been reported by studies among treated patients (Bensimon et al., 1994; Lacomblez et al., 1996; Riviere et al., 1998). Another alternative potential treatment is provided in the form of carboxyfullerenes, which are antioxidants, and operate by blocking glutamate receptor-mediated excitotoxicity (Dugan et al., 1997).

A glutamate analog, β-N-oxalyl-L-α, β-diaminopropionate (β-ODAP or BOAA), which is found in chickpeas, is believed to be responsible for upper motor neuron degeneration in lathyrism (Rothstein et al., 1992). A related toxin which is found in the cycad fruit, β-methylamino-L-alanine (BMAA), has been implicated in the ALS syndrome of Guam, although this theory has recently been brought into question because of the low level of consumption and long latent period (Meldrum, 2000). All these data provide support to the glutamate toxicity model.

Even though there is strong evidence for a link between glutamate excitotoxicity and ALS it is difficult to comprehend how the fast action of the glutamate receptors and transporters corresponds to very slow and progressive nature of damage to motor neurons brought about by the disease. A process may exist which chronically triggers glutamate release (Smith et al., 1995). Alternatively, the astrocytes expressing EAAT2 may play a role in mediating this damage. Early indicators of disease in ALS are astrocytic inclusions that stain with SOD1 and ubiquitin antibodies and which increase with the decrease of EAAT2 (Bruijn et al., 1997). In transgenic mouse models, damage to astrocytes is mediated by SOD1 mutants, which may lead to a reduction of EAAT2 levels and account for motor neuron degeneration (Bruijn et al., 1997). Additional support for this comes from astrocytes, which have been shown to interact directly with degenerating motor neurons via a process, which occurs during the early stage of disease involving mitochondrial vacuolation of motor neurons (Levine et al., 1999). Recently a study investigated further into the active participation of astrocytes in ALS by selectively expressing mutant SOD1 in astrocytes in a

mouse model of disease. This restriction of mutant SOD1 was enough to bring about astrocytosis but not degeneration of motor neurons (Gong et al., 2000).

### 1.5.3 Apolipoprotein E

The association of apolipoprotein E (APOE) alleles with Alzheimer's disease initiated the investigation of these alleles in ALS. The frequencies of apolipoprotein E (ApoE) ε2, ε3 and ε4 genotypes in familial and sporadic ALS do not vary from those in the general population (Mui et al., 1995), thereby excluding ApoE mutations as a major cause of ALS. However in some studies, the ApoE genotype has been reported to have an effect on the age of onset and clinical presentation of sporadic ALS. The ε3/ε3 genotype may extend survival in patients suffering from ALS, whereas the ε4 allele may predict an earlier age at onset (Al-Chalabi et al., 1996). In comparison, a significantly higher proportion of ALS patients with the ε3/ε4 genotype had bulbar symptoms, while the patients with ε2/ε3 genotype appeared to be predisposed to limb onset. Therefore, as in Alzheimer's disease, it has been suggested ε2 and ε3 alleles are protective in ALS, while the ε4 allele may play a deleterious role (Warwick Daw et al., 2000; Drory et al., 2001; Strittmatter et al., 1993).

The exact mechanism of the deleterious effect of the ε4 allele remains unknown, but it may be the result of the absence of the protective allele's ε2 and ε3. The gene products of APOE ε2 and ε3 genes have cysteine residues, essential for detoxification of lipid peroxidation product 4-hydroxy-nonenal (HNE), which triggers apoptosis and is increased in the spinal cord of patients suffering from ALS. The ε4 gene product has no cysteine residues, and as a result lacks the ability to protect against HNE-mediated apoptosis. This may be a possible explanation for the correlation between these APOE genotypes and susceptibility to neurodegeneration (Pedersen et al., 2000). However, other studies of ApoE in ALS have not confirmed the association of the ε4 allele with early-onset, shortened survival or the presence of bulbar symptoms at onset (Mui et al., 1995; Bachus et al., 1997; Smith et al., 1996). In addition, studies in Guam of patients with the ALS-Parkinsonism-dementia-complex (ALS-PDC) found no association with any of the ApoE alleles (Chen et al., 1996; Waring et al., 1994).

### 1.5.4 Ciliary neurotrophic factor (CNTF)

A reduction in the levels of ciliary neurotrophic factor (CNTF), a survival factor in spinal motor neurons, may play a role in the development of ALS. This conclusion is based on several lines of evidence. First, a decrease in the levels of CNTF was found in the corticospinal tract neurons of patients with ALS (Ono et al., 1999). Second, abolition of CNTF gene expression in knockout mice results in progressive motor neuron degeneration (Masu et al., 1993). Third, the frequency of the homozygous state for a mutation in the CNTF gene, resulting in absence of CNTF, is slightly increased in patients with sporadic ALS (Orrell et al., 1995). Since there is no increase in heterozygosity for CNTF mutations in ALS patients, the role of CNTF in ALS seems modest (Orrell et al., 1995; Takahashi et al., 1994).

### 1.5.5 Cytochrome P450 debrisoquine hydroxylase (CYP2D6)

Protein polymorphisms in the gene encoding cytochrome P450 debrisoquine hydroxylase (CYP2D6) are responsible for the reduced metabolism of exogenous toxins, thereby forming one of the risk factors associated with Parkinson's disease (Smith et al., 1992). Three studies have been carried out to investigate the potential role of the CYP2D6(b) allele as a risk factor for ALS. One study of 57 ALS patients and controls, found no association between this allele and ALS (James et al., 1994). The second study reported a considerable increase in the frequency of the CYP2D6(b) allele in 50 ALS patients (Siddons et al., 1996). The frequency of this mutant allele is higher in the Chamorro people than in Caucasians, although no differences have been found between healthy Chamorro people and patients with Guamanian ALS-PDC (Chen et al., 1996).

### 1.5.6 Apurinic apyrimidinic endonuclease (APEX)

The apurinic/apurimidinic exonuclease (APEX nuclease) gene encodes a multifunctional DNA repair enzyme, thought to be a risk factor for ALS, with reduced levels of APEX nuclease found in the frontal cortex of patients with ALS (Kisby et al., 1997). There are two reports of APEX gene mutations which lead to a decrease in the level and

activity of the enzyme in ALS: mutations were found in four of 117 patients with sporadic ALS and in four of nine twins with ALS, but not in controls (Olkowski et al., 1998; Hayward et al., 1999; Tomkins et al., 2000). APEX nuclease may also play a part in the Guamanian ALS-PDC, as a metabolite of cycasin, methylazoxy-methanol (MAM), a potential environmental genotoxin in Guamanian ALS found to cause a considerable reduction in neuronal APEX levels and activity (Esclaire et al., 1999). Though APEX mutations do not account for a significant proportion of ALS, the role of DNA damage in etiology of ALS may be importance.

### 1.5.7 Mitochondrial metabolism

There is accumulating evidence that inherited or acquired mitochondrial dysfunction contributes to the pathogenesis of ALS (Beal et al., 2000). Evidence of mitochondrial dysfunction has been found in muscle biopsies of patients with sporadic ALS (Wiedemann et al., 1998), and increased oxidative damage has been found in the central nervous system tissue from autopsied sporadic and familial ALS patients (Pedersen et al., 1998; Shaw et al., 1995; Bowling et al., 1993).

One of the earliest pathological findings in SOD1-mutant transgenic mice is mitochondrial vacuolization, suggesting that the leakage or translocation of mutant SOD1 in mitochondria may be the initial event sparking further degeneration (Dal Canto et al., 1994; Jaarsma et al., 2001; Kong et al., 1998; Wong et al., 1995). Mutations in mitochondrial (mt)DNA, such as depletion of mtDNA or multiple deletions of mtDNA (Vielhaber et al., 2000), as well as a mutation of the mtDNA-encoded subunit 1 of cytochrome c oxidase, have been described in ALS (Comi et al., 1998). Mitochondrial dysfunction in ALS may additionally result from decreased levels of mitochondrial manganese superoxide dismutase (SOD2); encoded by a nuclear gene on chromosome 6. An Ala9Val mutation in SOD2 has been detected in some patients with sporadic ALS (Van Landeghem et al., 1999), while SOD2 knockout mice have been found to exhibit degeneration of neurons in basal ganglia and brainstem, and progressive motor disturbances with paresis (Melov et al., 1998; Lebowitz et al., 1996). Furthermore, cultured murine cortical neurons with reduced levels of SOD2 show greater sensitive to glutamate neurotoxicity compared to neurons with intact

SOD2 activity (Li et al., 1998). In SOD1 transgenic mice, dysfunction of SOD2 exacerbates disease expression (Andreassen et al., 2000).

### 1.5.8 Selectivity of Cell Death in ALS

One of the unanswered questions with the regards to the study of ALS concerns the highly selective cell death of motor neurons. Figure 1.2 provides an overview of the motorneurone degeneration process in amyotrophic lateral sclerosis. Investigations have shown that with extended patient care degeneration of the cortex may take place (Takahashi et al., 1997), in addition a number of ALS cases have displayed (Bruijn et al., 1997) astrocytic damage, suggesting a more broad-ranging degeneration than previously thought. However, the majority of research has focused on those characteristics of motor neurons, which increase their vulnerability to damage.

As mentioned previously, SOD1 is a ubiquitously expressed protein and no alteration is observed in its abundance in motor neurons. However, a reduction in the activity of molecular chaperones is observed in mutant SOD1 cells and is thought to result in the accumulation and inefficient removal of mutant SOD1 (Bruening et al., 1999). Induction of stress-inducible heatshock proteins prevents the formation of mutant SOD1 aggregates suggesting reduced levels of available chaperones leaves mutant expressing SOD1 cells vulnerable to both physiological and environmental stress (Bruening et al., 1999). Alternatively, the high level of mitochondrial activity required by motor neurons and high neurofilament content, as a result of their large size and the ratio of cell body to axonal length, may render motor neurons selectively vulnerable. In sporadic ALS cases and controls, analysis of a number of physiochemical properties of NF failed to find distinctions in the purified protein (Strong, 1999). Abnormalities of collagen have been described in the spinal cord of ALS patients and implicated in selective vulnerability (Ono et al., 1998).

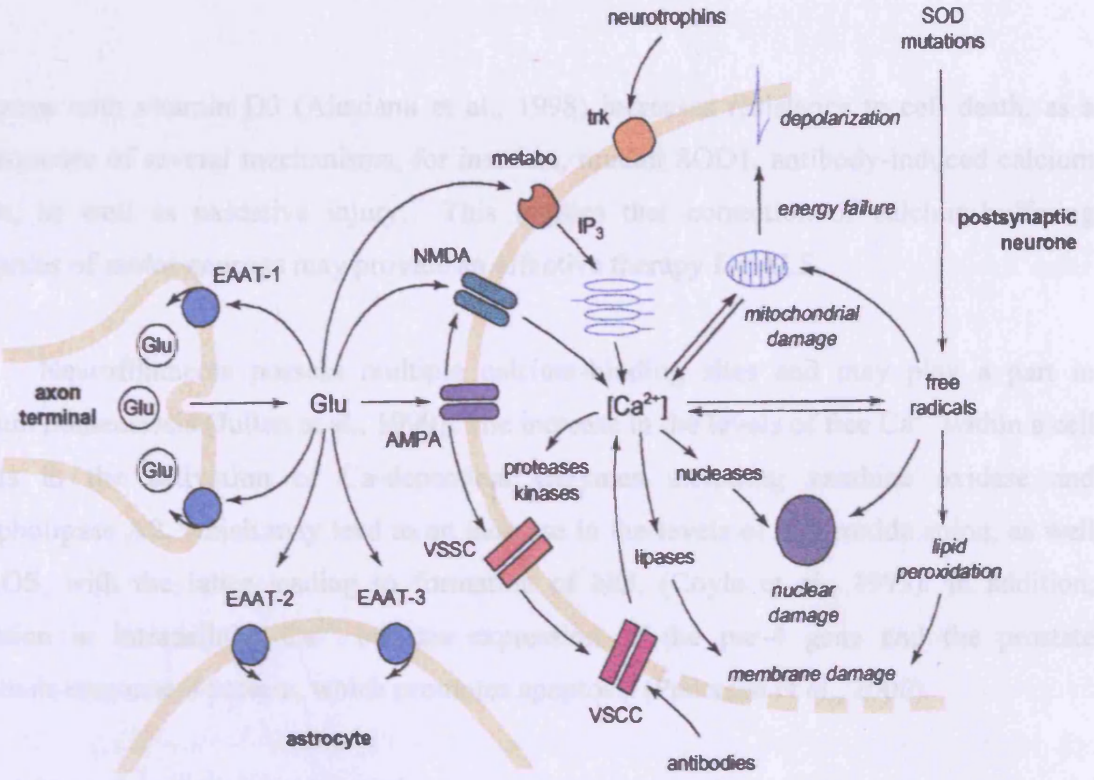



**Figure 1.2** A schematic representation of the biochemical systems potentially involved in the process of motoneurone degeneration. α-Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors, N-methyl-D-aspartate (NMDA) receptors and metabotropic receptors (metabo) are the three main subtypes of excitatory amino acid receptor. The three glutamic acid transporters are EAAT-1, EAAT-2 and EAAT-3. Abbreviations: IP3, inositol (1,4,5) trisphosphate; SOD, superoxide dismutase; trk, tyrosine receptor kinase; VSCC, voltage-dependent $Ca^{2+}$ channels: VSSC, voltage-dependent $Na^{+}$ channels. Diagram adapted from Louvel et al., (1997).

There is support for an increase in calcium levels, mitochondrial volume, as well as numbers of synaptic vesicles within the motor nerve terminal of ALS patients (Siklos et al., 1996). A deficiency in calcium-binding proteins such as calbindin D28k and parvalbumin, specific to cells damaged early in ALS, has been put forward as the reason for their particular vulnerability (Alexianu et al., 1994). The toxicity of SOD1 mutants is calcium-dependent and the selective death of motor neurons has been proposed to be the result of a combination of both calcium entry during the course of neurotransmission and the deficiency of Ca-binding proteins (Roy et al., 1998). Increased expression of calbindin D28k in motor neuron cells through viral infection (Ghadge et al., 1997; Ho et al., 1996) or

treatment with vitamin D3 (Alexianu et al., 1998) increases resistance to cell death, as a consequence of several mechanisms, for instance, mutant SOD1, antibody-induced calcium influx, as well as oxidative injury. This implies that correction of calcium-buffering properties of motor neurons may provide an effective therapy for ALS.

Neurofilaments possess multiple calcium-binding sites and may play a part in calcium homeostasis (Julien et al., 1999). The increase in the levels of free $Ca^{2+}$ within a cell results in the activation of Ca-dependent enzymes including xanthine oxidase and phospholipase A2, which may lead to an increase in the levels of superoxide anion, as well as NOS, with the latter leading to formation of NO, (Coyle et al., 1993). In addition, elevation in intracellular $Ca^{2+}$ induces expression of the par-4 gene and the prostate apoptosis response-4 protein, which promotes apoptosis (Pedersen et al., 2000).

The subunit GluR2 of AMPA glutamate receptor confers calcium impermeability to the receptor. Investigations have revealed human spinal motor neurons lack detectable levels of GluR2 mRNA, suggesting on these particular cells the AMPA receptors are calcium-permeable (Williams et al., 1997). However, reports have determined that rather than complete lack of GluR2, a subset of AMPA receptors has intermediate calcium permeability levels (Greig et al., 2000). Therefore there is no overall change in the relative levels of GluR2 or $Ca^{2+}$ permeability of AMPA receptors between motor neurons and those neuronal cells not affected by ALS (Vandenberghe et al., 2000). Furthermore, *in vitro* studies in motor neurons and other cells also expressing AMPA/KA $Ca^{2+}$ channels, comparing the effects of short-term exposure to AMPA and kainite, demonstrate the specificity of the stimulant and the reaction of the cell is distinct in motor neurons (Carriedo et al., 2000). The variation in desensitization of AMPA receptor in motor neuron cannot explain the selective vulnerability to injury. Instead, the susceptibility of motor neurons appears to be a result of higher density of functional AMPA receptors, which when pharmacologically reduced to levels observed in other cell types, eradicates the selective vulnerability to AMPA receptor agonists (Vandenberghe et al., 2000).

The mitochondria are the principal target of this calcium-mediated cellular damage. A large proportion of the cell's free radicals are generated by this organelle and it is

estimated, in the course of electron transfer to molecular oxygen, approximately 1-5% of electrons are lost and go on to form superoxide anions (Green et al., 1998). A critical role is played by mitochondria in the regulation of apoptosis and a correlation exists between increases in mitochondrial calcium and increases in free radicals associated with cell death (Beal, 2000; Stout et al., 1998).

A decrease in the activity of mitochondrial DNA (mtDNA)-encoded cytochrome c oxidase (cox) has been described in sporadic ALS cases. The same observation has been made in muscle biopsies from individuals with defects in mtDNA, suggesting that mitochondrial-encoded genes may play a role in the disease. The reduction in the activity of cox may play a part in neuronal death and come about as a result of oxidative damage to mtDNA (Borthwick et al., 1999). Alterations in mtDNA which arise as a result of decreased levels of membrane-associated mitochondrial SOD has been shown to account for the reduction in cox activity, suggesting defects in mtDNA are significant in sporadic ALS cases (Dhaliwal et al., 2000; Vielhaber et al., 2000). Chronic inhibition of mitochondria through complex II and IV blockers leads to selective motor neuron death. Prevention of cell death in this *in vitro* model is through treatment with free radical scavengers, AMPA/kainate receptor blockers, riluzole, and caspase inhibitors (Kaal et al., 2000). Even though the basal levels of oxidative metabolism are unchanged in ALS patients, once challenged with a stressful condition, the ability to compensate is reduced (Curti et al., 1996). In the course of neurotransmission, there is also a release of $Zn^{2+}$ at the presynaptic terminal. The AMPA receptors are $Zn^{2+}$-permeable, thereby enabling its entry into the cell where it can result in generation of ROS (Sensi et al., 1999). Mitochondrial metabolism is sensitive to levels of $Zn^{2+}$ and it is thought it may have a more potent effect than calcium (Weiss et al., 2000).

Mitochondrial DNA is a lot more sensitive than nuclear DNA to oxidative damage because of the lack of protective histones and efficient repair mechanisms. Neurons are more vulnerable to mtDNA damage because of their very high-energy requirements and the accumulation of damage to mtDNA may to some extent explain the progressive nature of the disease. The apoptotic cascade may be triggered by a reduction in the integrity of the mitochondria thereby resulting in cell death.

In a recent review of the advances in ALS research, the possibility of a pathway combining several of the proposed mechanisms in motor neuron death was proposed. Briefly, glutamate transport can be affected in a number of ways, for instance, by oxidative stress, alterations in EAAT2, or antibodies to voltage-gated calcium channels. Once activated, receptors bring about an influx in $Ca^{2+}$, which is aggravated as a result of the lack of calcium-binding proteins, and which results in disturbances in mitochondrial metabolism as well as the activation of a number of enzymes such as NOS. Nitric oxide spontaneously reacts with superoxide anion to generate peroxynitrite, which nitrosylates and consequently damages proteins (Al Chalabi et al., 2000). An excess of NO may also lead to an increase in production of $O^{2\cdot-}$ by inhibition of mitochondrial electron flow, thereby resulting in further generation of $ONOO^-$ (Torreilles et al., 1999).

The nitrosylation reaction is copper-dependent with the likely source of free copper being mutant SOD1, even in the presence of CCS. The target proteins for nitrosylation include the neurofilament subunits, which may lead to abnormal accumulation of NF and the subsequent disruption of the neurofilament network and axonal transport. In the presence of increased peripherin and decreased NF-L this reaction is enhanced (Al Chalabi et al., 2000). Mutant SOD1 has increased copper-dependent peroxidase activity, which results in the formation of hydroxyl radical that can also act on the NF network. It is logical to assume that disturbances of any of the molecules at any point in this complicated and at present incomplete pathway might lead to the cell death seen in ALS.

## 1.6 Cell Death and Apoptosis in Amyotrophic Lateral Sclerosis

The work described in this thesis utilises a model neuronal cellular system where apoptosis is a key feature and both Hsp27 and Hsp70 have a protective anti-apoptotic effect. As apoptosis is inhibited by Hsps, it is therefore necessary that this section provide a brief overview of the molecular pathways of programmed cell death (intrinsic and extrinsic pathways), before mentioning Hsps. In addition the mechanism by which this physiological process, which acts in all multicellular organisms, contributes to neurodegeneration and the role of this process in ALS will be discussed. It should be noted that the term- programmed

cell death (PCD) is a physiological process in which molecular programs that are intrinsic to the cell are activated to trigger its destruction; the term is frequently used interchangeably with 'apoptosis' which is only one morphological form of PCD (Clarke, 1990) characterized by membrane blebbing, cell shrinkage, nuclear condensation and DNA fragmentation. The following sections, briefly discuss the molecular composition of the PCD machinery, followed by PCD in ALS and how targeting of PCD has led to the disclosure of molecular targets for the development of drugs for prevention and treatment of neurodegenerative diseases and ALS in particular.

### 1.6.1 Molecular pathways of Programmed Cell Death (PCD)

The principal mediators of programmed cell death are the proteolytic enzymes known as 'caspases', which cleave their substrates after specific aspartic acid residues. Caspases exist in nearly every animal cell as zymogens known as 'procaspases', and become activated in response to intracellular signalling pathways initiated by various cellular signals, such as DNA damage and withdrawal of trophic support. In total, the family of mammalian caspases comprises 15 members, which can be divided into initiators (procaspases 2, 8, 9 and 10) and effectors (procaspases 3, 6 and 7) of PCD pathway. The first caspases to become activated in the PCD cascade are the initiator caspases. The initiator caspases possess long amino-terminal prodomains that contain specific protein-protein interaction motifs. It is through these domains, that initiator caspases 8 and 9 are activated after being aggregated by the adaptor molecules FADD (Fas-associating protein with death domain); (Muzio et al., 1998) and Apaf1 (apoptotic protease-activating factor 1); (Li et al., 1997), respectively. The activated initiator caspases subsequently cleave effector procaspases into their active forms, which are responsible for events such as mitochondrial damage, nuclear membrane breakdown, DNA fragmentation, chromatin condensation and, eventually, cell death.

The Bcl2 family of proteins, which are implicated in the regulation of PCD, contain members that have either anti-PCD (for instance Bcl2 and Bcl-xL) or pro-PCD (for instance Bax and Bak) acting (Gross et al., 1999). The members all share some degree of structural similarity and can have up to four Bcl2-homology domains (BH1-BH4). In addition to the

many Bcl2 members that contain BH domains, such as Bcl2 per se and Bax, there exist molecules that share sequence homology only with the BH3 domain, such as Bid or Bim. These BH3 only proteins function as intracellular death ligands, proximal to multidomain Bcl2 members, and additionally can connect with proximal signal transduction pathways (Letai et al., 2002). Multidomain Bcl2 members can regulate the release of mitochondrial apoptogenic factors such as cytochrome c, Smac/Diablo, or apoptosis-inducing factor (AIF) and as a result can preserve or disrupt mitochondrial integrity. In addition, Bcl2 can also inhibit initiator caspases by means of mitochondrial independent mechanism (Marsden et al., 2002).

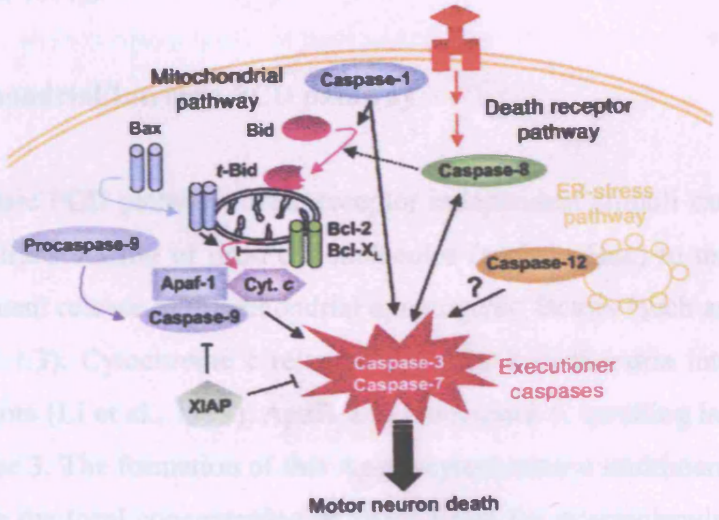



**Figure 1.3** The molecular pathways involved in the process of Programmed Cell Death (PCD). Three different PCD molecular pathways have been recognized: the mitochondrial pathway (also called intrinsic), the death receptor pathway (also called extrinsic), and the ER pathway. Diagram adapted from Guégan et al., (2003).

### 1.6.1.1 The death receptor/Extrinsic PCD pathway

The extrinsic PCD pathway (figure 1.3) is triggered on activation of cell surface death receptors such as Fas/CD95 and the tumour necrosis factor receptor 1 (TNFR1).

Activation of death-receptor is produced by specific ligands known as death activators, for instance, the Fas ligand binds to Fas, and TNFα binds to TNFRl. On binding, the intracellular 'death domains' on these receptors associate with the 'death effector domains' on an adaptor protein, for instance, Fas associates with FADD, and TNFR1 associates with FADD and TRADD (TNFR-associated protein with death domain). Adaptor proteins then recruit pro-caspase 8, and result in its activation. Activated caspase 8 triggers the activation other caspases, either directly or indirectly, through cleavage of Bid (fig.1.3). The extrinsic PCD pathway is particularly instrumental in pathological conditions in which inflammation is a prominent characteristic. In addition to being activated by the extrinsic pathway, it appears that procaspase 8 is also cleaved by a mechanism that depends on the intrinsic pathway (Slee et al., 1999).

### 1.6.1.2 The mitochondrial/Intrinsic PCD pathway

In the intrinsic PCD pathway, death receptor independent stimuli can instigate PCD by bringing about translocation of pro-PCD molecules (such as Bax) to the mitochondria, resulting in subsequent release of mitochondrial apoptogenic factors (such as cytochrome c) to the cytosol (fig. 1.3). Cytochrome c released from the mitochondria interacts with two other cytosolic factors (Li et al., 1997), Apaf1 and procaspase 9, resulting in the subsequent activation of caspase 3. The formation of this Apaf1-cytochrome c multimeric complex may function to increase the local concentration of procaspases for intermolecular cleavage, and to set a relatively high threshold of caspase activation in order to ensure an occasional leakage of cytochrome c does not trigger PCD (Budihardjo et al., 1999).

Another mitochondrial inter-membrane protein that is released into the cytosol on induction of PCD is Smac/Diablo (Du et al., 2000; Verhagen et al., 2000). Once in the cytosol, Smac/Diablo interacts with several inhibitors of apoptosis (IAPs), thereby reducing the inhibitory effect of IAPs on initiator (such as caspase 9) and effector caspases (such as caspase 3) (Chai et al., 2000; Srinivasula et al., 2000). In contrast to cytochrome c and Smac/Diablo, the release of AIF and endonuclease G from the mitochondrial intermembrane space does not result in caspase activation (Susin et al., 1999; Li et al., 2001). Under certain death-inducing stimuli, AIF translocates from the mitochondria to the nucleus where it

triggers large-scale DNA fragmentation through caspase-independent mechanisms (Susin et al., 1999). In the same way, endonuclease G, which is normally involved in the replication of mitochondrial DNA, can translocate to the nucleus on induction of PCD, and as a result bring about fragmentation of nuclear DNA (Li et al., 2001). The AIF homologue wah-1, in *Caenorhabditis elegans* has been described to associate and cooperate with endonuclease G to promote DNA degradation and apoptosis (Wang et al., 2002).

Stress in the endoplasmic reticulum (ER), such as disturbance of calcium homeostasis and accumulation of unfolded proteins, can also result in PCD (Nakagawa et al., 2000) through activation of caspase 12; which in turn can cleave caspase 9 (Morishima et al., 2002). Pro-PCD members of the Bcl2 family – for instance Bax and Bak - function at the ER to preserve calcium homeostasis as well as regulate ER-dependent PCD (Scorrano et al., 2003).

### 1.6.2 Programmed Cell Death in Amyotrophic Lateral Sclerosis

Early on in the effort to ascertain the nature of mutant SOD1's gained toxic function, it was discovered that transfected neuronal cells expressing mutant SOD1 cDNA were dying by apoptosis (Rabizadeh et al., 1995). Subsequently similar observations were made in transfected PC-12 cells (Ghadge et al., 1997) and in primary neurons obtained from transgenic mice expressing mutant SOD1 (Mena et al., 1997). All these *in vitro* data have led many investigators to believe that mutant SOD1 may kill motor neurons by activating PCD; a term used for cell death mediated by specific signalling pathways. Ever since the identification of neuronal apoptosis inhibitory protein (NAIP) as a candidate gene for an inherited ALS-related disorder known as spinal muscular atrophy, the possible implication of PCD in ALS has been rather appealing to the field of motor neuron diseases (Roy et al., 1995).

### 1.6.3 Morphology of dying motor neurons

In light of the acknowledged proapoptotic properties of mutant SOD1 observed *in vitro*, it may be wondered whether, dying spinal cord motor neurons of transgenic mutant

SOD1 mice, also present features of apoptosis, whose morphological hallmarks include cytoplasmic and nuclear condensation, compaction of nuclear chromatin into sharply circumscribed masses along the inside of the nuclear membrane, and preservation of structure of organelles (at least up until the cell is broken into membrane-bound fragments called apoptotic bodies that are phagocytosed). This question has been investigated in several morphological studies carried out in transgenic mutant SOD1 mice (Dal Canto et al., 1995; Migheli et al., 1999; Pasinelli et al., 2000; Vukosavic et al., 2000). Most of the ailing neurons are atrophic, in these animals, and their cytoplasm is dominated with vacuoles corresponding to dilated rough ER, Golgi apparatus, and mitochondria (Dal Canto et al., 1995). Ultrastructural studies in these mice (Guégan et al., 2003) indicate that the ailing neurons have diffusely condensed cytoplasm and nuclei, along with irregular shapes. At present, the actual type of cell death remains to be determined.

Clarke (1999) reported the dying neurons display non-apoptotic morphology. However Guégan et al. (2003) were able to detect apoptotic cells in the spinal cord of transgenic mutant SOD1 mice and estimated that in end-stage transgenic $SOD1^{G93A}$ mice, which have lost about 50% of their anterior-horn motor neurons, about two apoptotic cells will be seen per 40μm-thick section of the lumbar spinal cord. It has been observed that the vast majority of these apoptotic cells no longer display definite morphological characteristics or express phenotypic markers that enable their identification as neurons or glia (Guégan et al., 2003). However, less than 15% of the spinal cord apoptotic cells are still immunoreactive for specific proteins such as neurofilament or glial fibrillary acid protein (Pasinelli et al., 2000), which suggests that in the mutant SOD1 model, both neuronal and glial cells are dying by apoptosis (Vukosavic et al., 2000). The rareness in this mouse model of ALS, of apoptotic motor neurons, reflects the difficulty in detecting these cells by morphological means due to the supposed low daily rate of motor neuron loss (Chiu et al., 1995) and the extremely rapid clearance of apoptotic bodies. Forms of PCD exist which possess morphological features distinct from apoptosis (Clarke, 1999; Yaginuma et al., 1996; Sperandio et al., 2000), making it difficult to rule out the possibility that a non-apoptotic form of PCD triggers mutant SOD1-related cellular degeneration.

Using morphological criteria such as size, shape and aggregates of Nissl substance, in human ALS cases, Martin (Martin, 1999) has arranged residual spinal cord motor neurons in ALS post-mortem samples in three categories that he considers reflect various stages of degeneration. In the chromatolysis stage, motor neurons still look like their normal counterparts apart from the fact that the cell body appears swollen and round, the Nissl substance dispersed, and the nucleus unconventionally placed. Prominent cytoplasmic hyaline body inclusions can be seen in some chromatolytic neurons. In the attritional stage, the cytoplasm and the nucleus both appear homogenous and condensed, whereas the cell body which has a hazy multipolar shape appears shrunken. In the so-called apoptotic stage, the affected motor neuron is approximately one-fifth of its typical diameter, with the cytoplasm and nucleus both extremely condensed, and the cell body has a fusiform or round shape devoid of any process. In particular, in none of these three stages do residual motor neurons exhibit a significant number of cytoplasmic vacuoles or nuclear condensation accompanied by round chromatin clumps. Collectively, these findings propose that, while degenerating neurons in both human ALS and its experimental models do display some characteristics reminiscent of apoptosis, the vast majority of dying cells cannot be labelled confidently as typically apoptotic.

### 1.6.4 Expression of apoptotic markers

In addition to exhibiting singular morphological features, apoptotic cells may also display a variety of cellular alterations. Detection of internucleosomal DNA cleavage by means of either gel electrophoresis or in situ methods has emerged as a popular means of providing evidence for the occurrence of apoptosis in all kinds of pathological situations, including ALS. However, like many of these apoptotic markers, DNA fragmentation detected by in situ methods, such as terminal deoxynucleotidyl transferase-mediated nick end labelling, is now well recognized as also taking place in nonapoptotic cell death, including necrosis (Clarke, 1999). Therefore the value of DNA cleavage as a specific marker of apoptosis, evidenced by in situ techniques, may be limited. In addition to this limitation, conflicting results have been obtained from the search for DNA fragmentation in ALS post-mortem samples.

In one autopsy study, an *in situ* method was used to detect DNA fragmentation in spinal cord motor neurons, which gave positive results in ALS and negative in control specimens (Yoshiyama et al., 1994). In two other similar studies, fragmentation of DNA was not only detected in the motor cortex and spinal cord of ALS specimens, but also, though to a lesser extent, in control specimens (Ekegren et al., 1999; Migheli et al., 1994.). A subsequent study, detected internucleosomal fragmentation of DNA in affected (e.g., motor cortex and spinal cord) but not in spared brain regions (e.g., somatosensory cortex) of ALS cases (Martin, 1999), as well as in diseased motor neurons, only at the somatodendritic attrition and apoptotic stages but not at the chromatolytic stage (Martin, 1999). The author of that study has also documented DNA fragmentation, by gel electrophoresis, in anterior-horn gray matter of the spinal cord and motor cortex of ALS cases (Martin, 1999), a technique not commonly used to identify apoptosis in the nervous system simply because in many neurological situations, it is difficult to obtain samples with a sufficiently high proportion of dying cells. In contrast to all these positive findings, other groups, utilising similar techniques and tissue samples, have been unsuccessful in providing any evidence of internucleosomal DNA cleavage in post-mortem tissue from human ALS cases or from animal models of the disease (Migheli et al., 1994 & 1999; He et al., 2000). Though the actual reason for these conflicting results is unclear, the reliability and even the specificity of such findings are brought into question.

Two other apoptotic markers also studied in ALS, include the $Le^Y$ antigen (Hiraishi et al., 1993) and fractin (capase-cleaved actin) (Suurmeijer et al., 1999), which make the picture less ambiguous. Hiraishi et al. (1993) demonstrated that the expression of the carbohydrate $Le^Y$ antigen, which results from the co-operative action of the fucosyltransferases $\alpha$1(r)2 and $\alpha$1(r)3, showed good correlation with the presence of apoptosis in both normal and tumour tissue. However, not all $Le^Y$-positive cells showed signs of apoptosis and necrosis not associated with $Le^Y$ antigen expression. Neither of these markers was detected in spinal cords of controls but both were highly expressed in spinal cords of ALS cases (Yoshiyama et al., 1994) and transgenic $SOD1^{G93A}$ mice (Vukosavic et al., 2000). Similarly, increased levels of the apoptosis-related protein prostate apoptosis response-4 (Rangnekar, 1998) were detected in spinal cord samples from both ALS patients and transgenic mutant SOD1 mice compared to their respective controls (Pedersen et al.,

2000). Together with the morphological data summarized above, these findings provide support to the idea that apoptosis occurs in ALS. However, all of these studies fail to provide definite mechanistic insights into the significance of these alterations in the pathogenesis of ALS.

### 1.6.5 Activation of apoptotic molecular pathways

As the results of morphological studies remain unclear, it appears that a more realistic approach to assessing the role of apoptosis in ALS may be to ascertain whether the neurodegenerative process in transgenic mutant SOD1 mice, irrespective of the morphology of the dying cells, involves known molecular mediators of PCD, and whether targeting such crucial and key factors can affect the course of the disease.

PCD is a multistep mechanism that involves a complex interaction between survival pathways which are activated by trophic factors and death pathways, activated by various stresses. Thus far, two pathways have been most implicated in neuronal survival. One is the PI3K pathway, which activates Akt (also known as protein kinase B) to suppress the activation of proapoptotic proteins, and the other the extracellular signal-regulated kinase/MAPK (ERK/MAPK) pathway, which activates antiapoptotic proteins (Harper et al., 2001).

Fas appears to contribute to the death of embryonic motor neurons in primary cultures (Raoul et al., 2002), but whether it contributes to the death of mature motor neurons and to the process of neurodegeneration in transgenic mice expressing mutant SOD1 remains to be shown. Other important molecules in PCD signalling include ceramide, MAPKs (JNK and p38), and the transcription factors activator protein-1 and NF-κB (Harper et al., 2001; Gupta, 2001).

In light of the supposed proapoptotic properties of mutant SOD1 (Rabizadeh et al., 1995), it is attractive to suggest that the mutant protein may be a death-signalling molecule in itself, either by directly interacting and setting in motion the PCD cascade, or by indirectly interacting with a variety of intracellular targets such as trophic factors, Bcl-2

family members, or even mitochondria. Particularly appealing targets are the mitochondria, not only because they contain mutant SOD1 (SOD1) but because they are structurally and functionally altered in transgenic mutant SOD1 mice (Kong et al., 1998; Browne et al., 1998), and because they play a critical role in PCD (Kroemer et al. 2000). Of additional relevance to the issue of death and survival signals, in the mutant SOD1 model, are the Western blot and immunohistochemical demonstrations of the weakening of the surviving signal mediated by PI3K/Akt in spinal cords of transgenic mutant SOD1 mice, prior to the development of overt neuropathological features (Warita et al., 2001). Several secondary alterations arise in spinal cords of transgenic mutant SOD1 mice, on initiation of the mutant SODl-mediated neurodegenerative process, such as microglial cell activation (Almer et al., 1999) and T cell infiltration (Alexianu et al., 2001), both of which may release a surplus of cytokines and other pro-PCD mediators. Thus, while the nature of the initial death signal in transgenic mutant SOD1 mice remains indefinable, in a more advanced stage of the disease the increased expression of a number of extracellular inflammation-related factors such as IL-lβ, IL-6, and TNF-α (Nguyen et al., 2001) may result in the amplification of death signals that are already reaching motor neurons in this mouse model of ALS, by activating death receptors such as Fas (Raoul et al., 2002). Elevation in levels of IL-lβ also occurs in human ALS spinal cords (Li et al., 2000).

### 1.6.6 The role of the Bcl-2 family in motor-neuronal cell death in ALS

The Bcl-2 family, implicated in the regulation of PCD, is composed of both suppressors of cell-death for instance Bcl-2 and Bcl-xL and promoters such as Bax, Bad, Bak, and Bcl-xS (Chao et al., 1998). Within the nervous system several of these molecules are present and active and appear to be powerful modulators of neuronal death. Levels of Bcl-2 mRNA appear significantly decreased and Bax mRNA levels significantly increased in the lumbar cord compared with those of controls, in both human ALS cases and affected transgenic SOD1G93A mice (Mu et al., 1996; Vukosavic et al., 1999). This is consistent with the finding in both human ALS cases and symptomatic transgenic $SOD1^{G93A}$ mice, that the expression levels in the spinal cord of the antiapoptotic proteins Bcl-2 and Bcl-xL are either unchanged (Ekegren et al., 1999; Troost et al., 1995) or decreased (Martin, 1999; Vukosavic et al., 1999), compared to expression levels of the proapoptotic proteins Bax and

Bad which increase (Martin, 1999; Ekegren et al., 1999; Vukosavic et al., 1999). More importantly, it should be noted that a very similar pattern of changes in expression of selected pro- and anti-cell death Bcl-2 family members was found in spinal cords of affected transgenic $SOD1^{G86R}$ mice compared with their wild-type counterparts (Gonzalez de Aguilar et al., 2000). However, none of these changes are observed in young asymptomatic transgenic $SOD1^{G93A}$ mice, but they clearly become more prominent as the neurodegenerative disease process progresses (Vukosavic et al., 1999). Regarding function, Bax is not only upregulated but is also expressed primarily in its active homodimeric conformation, in the spinal cords of both ALS patients and affected transgenic mice expressing mutant SOD1 (Martin, 1999; Vukosavic et al., 1999). Bax is markedly relocated from the cytosol to the mitochondria (Martin, 1999; Guégan et al., 2001; Przedborski et al., 2003); this relocation is, under many cellular conditions, a prerequisite to the employment of the mitochondria-dependent apoptosis pathway.

During the neurodegenerative process in ALS, it appears as though the fine-tuned balance between cell-death antagonist and agonist of the Bcl-2 family is upset in favour of pro-cell death forces. This view is supported by the finding that overexpression of Bcl-2, probably by buffering some of the pro-cell death force (Vukosavic et al., 1999), alleviates neurodegeneration and hence prolongs survival in transgenic $SOD1^{G93A}$ mice (Kostic et al., 1997); in addition to this a similar beneficial effect of Bcl-2 was reported in mutant SODl-transfected PC-12 cells (Ghadge et al., 1997).

Two other important members of the Bcl-2 family, which appear to play a role in ALS, are Bid and Harakiri. These two potent pro-PCD peptides participate in the cell death process, either directly or indirectly, by modulating the effect of Bax. Bid appears to be highly expressed in the spinal cord of transgenic $SOD1^{G93A}$ mice and attains its most active form following cleavage, during the progression of the disease (Guégan et al., 2002). Harakiri's expression has been detected specifically in spared motor neurons of ALS spinal cord specimens but not of control, some of which exhibited an abnormal morphology reminiscent of that labelled by Troost et al. (Troost et al., 1995) as apoptotic (Shinoe et al., 2001).

As in other pathological circumstances, it is doubtful that mutant SOD1 directly produces the observed changes in Bax. It is more likely that mutant SOD1 triggers intracellular signalling pathways, which consequently bring about Bax upregulation and translocation. This scenario would be consistent with what is currently known about the regulation of Bax and its activation is in PCD. The tumour suppressor protein p53 is one of the rare molecules known to regulate Bax expression (Miyashita et al., 1994). Under normal conditions, basal levels of p53 in the cell are very low, but once activated, as seen in pathological situations, there is a rapid increase in p53 mRNA and protein levels, as well as posttranslational modifications that stabilize the protein (Appella et al., 2001).

Evidence supporting activation of the p53 pathway in ALS comes from increased levels of p53 in the nuclear fraction of affected brain regions in ALS patients (Martin, 2000), along with increased levels of p53 immunostaining in neuron nuclei of transgenic $SOD1^{G86R}$ mice (Gonzalez de Aguilar et al., 2000). In spite of this compelling evidence that p53 is activated in ALS, two independent studies investigating the cross of G93A mice with p53-knockout mice, have been unsuccessful in providing any supportive data for an actual role for this transcription factor in mutant SODl-mediated neurodegeneration (Prudlo et al., 2000; Kuntz et al., 2000).

### 1.6.7 Caspases in the ALS neurodegenerative process

An instrumental role for caspases in ALS neurodegeneration is supported by the fact that benzyloxycarbonyl-Val-Ala-Asp(O-methyl)-fluoromethylketone, an irreversible broad-caspase inhibitor, attenuates mutant SODl-mediated cell death in transfected PC-12 cells (Ghadge et al., 1997) as well as in transgenic $SOD1^{G93A}$ mice (Li et al., 2000).

All of the identified caspases are placed in groups based on their function. One group, which is now thought to play a key role in cytokine maturation, includes caspases -1, -4, -5, -11, -12, and -14. Among this group the most attention, in ALS, has been given to caspase-1, which is the key enzyme responsible for the activation of IL-1. Procaspase-1 is expressed at high levels in spinal cord motor neurons, and its activation coincides with the development of the glial response and with the initial stage of motor neuron loss in the

spinal cord of transgenic mutant SOD1 mice (Pasinelli et al., 1998 & 2000; Vukosavic et al., 2000; Li et al., 2000; Guégan et al., 2001). In spite of the possible indirect role of caspase-1 in PCD, chronic inhibition of caspase-1 by means of a dominant negative mutant of the enzyme has been demonstrated to be effective in prolonging the life of transgenic $SOD1^{G93A}$ mice (Friedlander et al., 1997).

At present, the status of the other members of the caspase-1 subfamily is unknown in ALS. Some preliminary studies have shown that caspase-12, known to be activated following ER stress (Nakagawa et al., 2000), is expressed in motor neurons of nontransgenic mice, and to a greater extent in those of symptomatic transgenic $SOD1^{G93A}$ mice (Guégan et al., 2003). In symptomatic transgenic $SOD1^{G93A}$ mice, the majority of the motor neurons that stain immunopositive for caspase-12 appear condensed, shrunken, and vacuolized (Guégan et al., 2003) Although further work on caspase-12 remains to be carried out in this model of ALS, preliminary data suggests that sick cells represent the site of an ER stress whose occurrence may well play a role in the overall cascade of deleterious events that ultimately underlies the demise of spinal cord motor neurons in the mutant SOD1 model (Guégan et al., 2003). By contrast, caspases -2, -3, -6, -7, -8, -9, and -10 have been implicated in apoptosis; however their roles can be further sub-divided into "initiator" and "effector."

Interestingly, although significant glial response and IL-1β production takes place early in transgenic mutant SOD1 mice, activation of procaspase-8, just like the induction of TNF-α (Nguyen et al., 2001), is only detected near the end stage in the spinal cords (Guégan et al., 2002). This implies that, in this ALS model, TNF/caspase-8 may be a relatively late contributor to the process of degeneration. Another initiator of PCD is caspase-2, whose activation occurs in the spinal cord of affected transgenic mutant SOD1 mice (Guégan et al., 2003). However, caspase-2 ablation in transgenic $SOD1^{G93A}$ mice has been reported to make no impact to the expression of the disease (Bergeron et al., 1998), suggesting that whatever the role is of caspase-2 in ALS, it is not essential. Caspase-9 is a third caspase initiator, whose role is crucial in the so-called mitochondria-dependent PCD pathway (Kroemer et al., 2000). Evidence of a prominent role of this mitochondrial pathway has been documented in specimens of spinal cord of both ALS patients and transgenic $SOD1^{G93A}$ mice (Guégan et

al., 2001). The study showed that while cytochrome c is confined to the mitochondria in cells in the control samples, it is diffusely distributed in the cytosol in many of the spared cells, especially neurons in the pathological samples.

It has also been shown in transgenic mutant $SOD1^{G93A}$ mice that the mitochondrial cytochrome c translocation to the cytosol coincides with the translocation of cytosolic Bax to the mitochondria and activation of procaspase-9, prior to activation of downstream executioner caspases such as procaspase-3 and procaspase-7. As caspase-9 is believed to be so crucial under many cell-death situations, it is possible that the translocation of cytochrome c and activation of procaspase-9 observed in ALS may represent significant pathological events. The finding that prevention of mitochondrial cytochrome c release lengthens the lifespan of transgenic $SOD1^{G93A}$ mice is consistent with this view (Zhu et al., 2002).

Procaspases -3, -6, and -7 make up the effector caspases, all of which have short prodomains and lack intrinsic enzymatic activity. However, once effector caspases are cleaved, for example by initiator caspases, they acquire the capacity to cleave a large number of intracellular substrates, which probably eventually results in the demise of the cell. Consistent with this, it has been reported that key effector caspases such as caspase-3 and caspase-7 are indeed activated in spinal cords of transgenic mutant SOD1 mice in a manner that appears to be time-dependent and which parallels the time course of the neurodegenerative process (Pasinelli et al., 2000; Vukosavic et al., 2000); in addition, activation of procaspase-3 has also been observed in spinal cord samples from ALS patients (Martin, 1999). However, current data on the sequence of events in the PCD cascade suggest that, once effector caspases have been activated, the cell death process has reached a point of no return, at least under certain pathological situations. This suggests that, in these specific conditions, the death commitment point is situated upstream of these caspases, and as a result, interventions aimed at inhibiting these downstream caspases may be unsuccessful in providing any real neuroprotective benefit (Zheng et al., 2000). It remains to be determined whether this applies to the demise of motor neurons in ALS.

## 1.7 Towards the Development of ALS Therapies

ALS is among the many neurodegenerative diseases for which there is currently no cure. The following sections provide a short overview of what is currently available to patients suffering from ALS, what is being developed and the targets for the development of these new and hopefully more effective treatments. The use of viral vectors in the context of gene therapy is also discussed, followed by a description of the HSV-1 based vectors which were originally developed for in vivo gene delivery in the central nervous system and which were subsequently utilised in the work presented in this thesis.

### 1.7.1 Current treatment and potential therapies for ALS

To date, multiple clinical trials have been completed using individual agents including recombinant human ciliary neurotrophic factor (rHCNTF), riluzole, insulin-like growth factor-1 (IGF-1), topiramate, alpha-tocopherol (vitamin E), creatine, selegine, nimodipine, verapamil, and gabapentin (Bensimon et al., 1994; Borasio et al., 1998; Desnuelle et al., 2001; Jackson et al., 2001; Kwiecinski et al., 2001; Miller et al., 1996 & 2001; Cudkowicz et al., 2003; Groeneveld et al., 2003). Regrettably however, none of these single agents has produced significant benefit. Currently, as mentioned previously Riluzole is the only FDA-approved drug for ALS patients, and has a modest effect on survival, respiratory capacity and rate of declining strength, but not on total strength, quality of life, or functional capacity (Lacomblez et al., 1996). From data obtained from animal models, it is known that anti-glutamate agents are thought to affect the initiation but not the propagation of motor neuron degeneration; the converse being true for antioxidant agents (Gurney et al., 1996; Torreilles et al., 1999; Ochs et al., 2000; Simpson et al., 2003).

A combinational drug treatment that addresses different pathophysiologic mechanisms makes sense and is necessary to achieve the most significant benefit (Eisen et al., 1999; Mitsumoto et al., 2001; Carter et al., 2003). Several reports, utilising different laboratory models of motor neuron degeneration, support the benefit of combination drug therapy (Bilak et al., 2001; Iwasaki et al., 1999; Kriz et al., 2003; Mohammadi et al., 2001; Nagano et al., 1999 & 2003; Zhang et al., 2003). Some success has been obtained from

limited combination drug trials, suggesting synergistic effects between different drugs (Stevic et al., 1998; Cudkowicz et al., 2003). Recently rasagiline in combination with riluzole was found to be a promising clinical combination with survival being extended by approximately 20% in ALS mouse model (Waibel et al., 2004). In addition the combination of minocycline with riluzole was also shown to provide a greater neuroprotective effect than riluzole alone and currently phase III trial in ALS is currently underway (Gordon et al., 2004).

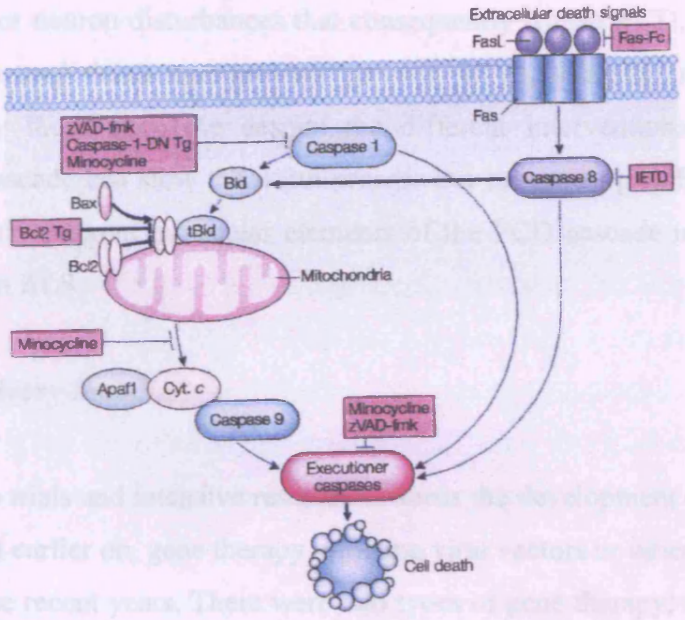



**Figure 1.4** Diagram shows how programmed cell death may be targeted in amyotrophic lateral sclerosis. In a transgenic mouse model of amyotrophic lateral sclerosis (ALS), initiator caspases activate downstream effector caspases primarily through the recruitment of the mitochondrial programmed cell death pathway. Interfering with different molecular elements of this cascade (red boxes), and particularly with caspase activation, has been shown to significantly delay disease onset and mortality in this experimental model of ALS. Abbreviations: Cyt., cytochrome; DN, dominant negative; FasL, Fas ligand; IETD Ile-Glu-Thr-Asp-fluoromethylketane; tBid, truncated Bid; Tg, transgenic; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone. Diagram adapted from Vila et al., (2003).

Inhibition of PCD, through use of pan-caspase inhibitor zVAD-fmk (Li et al., 2000) and through over-expression of Bcl-2 (Kostic et al., 1997), in transgenic mice that express mutant SOD1, delays but does not permanently prevent neurodegeneration, suggesting that

PCD may be triggered as a result of a direct effect, but from an indirect effect of the mutant protein on the PCD machinery (Guégan et al., 2003). In keeping with this view, mutant SOD1 has the susceptibility to form intracellular aggregates, the presence of which in the cytosol of motor neurons might impair microtubule dependent axonal transport (Cleveland et al., 2001) and tie-up important molecular chaperones (Bruening et al., 1999). It might therefore be possible that mutant SOD1, promotes protein aggregation, which then brings about important motor neuron disturbances that consequently trigger PCD. Interfering with PCD can delay neuronal death and prolong survival in experimental models of ALS. However, eventually these mice die despite the different interventions indicating that targeting the PCD cascade can slow the death process but cannot stop it. Figure 1.4 shows how interference with different molecular elements of the PCD cascade may significantly delay disease onset in ALS.

### 1.7.2 Viral gene delivery for ALS?

In addition to trials and intensive research towards the development of effective ALS treatments, described earlier on, gene therapy, utilising viral vectors or other means, has also been developed in the recent years. There were two types of gene therapy; the first involves the delivery of a functional gene to an individual who has a defective copy gene concerned and the second involves the delivery of genes, the protein products of which provide beneficial effect in treatment of symptoms of the disease concerned. In ALS the second approach has shown a certain degree of promise.

The strategy for the development of gene therapy procedures for ALS aims to deliver factors protective for the motor neuron or factors with neurorestorative properties in a safe and standardised manner. For example, delivery of genes encoding neurotrophic factors such as either IGF-1 (insulin-like growth factor 1) or GDNF (glial-derived neurotrophic factor), via engineered adeno-associated virus (AAV), to promote the survival of neurons (Kaspar et al., 2003).

Naturally occurring trophic factors that promote the survival and growth of motor neurons (Elliot et al., 1996; Oppenheim, 1996) have been attractive candidates for such a

therapy; however they have had a disappointing history in ALS. Driven mostly by hope and little more than suggestive pre-clinical evidence, trials carried out with ciliary neurotrophic factor and brain-derived trophic factor have been unsuccessful. Although IGF-1 brought about a mild slowing of disease in ALS patients in a North American trial (Lai et al., 1997) results of a European trial were negative (Borasio et al., 1998). The multiple failures have triggered a growing pessimism for the overall trophic strategy, although they could reflect the use of wrong neurotrophins or, even simply, failure to deliver the neurotrophin effectively to the target cells of the spinal cord.

HSV-1 has a number of properties which make it a good candidate for the development of vectors for the delivery of genes to the nervous system and hence development of ALS therapies: (a) natural tropism for neurons, (b) large ~150 kb viral genome allowing the insertion of multiple therapeutic genes <20 kb (c) ability to establish asymptomatic life-long latent infections. However, disadvantages include vector toxicity and an inability to maintain long-term transgene expression, as a result of latency-associated problems; studies to overcome these problems are ongoing.

Although many studies were reported to have delivered various genes to muscle and motor neurons of different animal ALS models and through utilising different viral vectors, at present the optimal vector remains uncertain. Examples of successful trials utilising viral gene delivery include the study conducted by Azzouz et al. (2000) in transgenic ALS mice demonstrating increased motoneuron survival and improved neuromuscular function following intraspinal injection of an AAV encoding Bcl-2. Kaspar et al. (2003) extended the lifespan of SOD1-G93A transgenic mice by approximately 5 weeks through viral deliver of IGF-1 via AAV, however similar delivery of GDNF was much less effective. Azzouz et al. (2004) showed that delivery of VEGF (vascular endothelial growth factor) via lentiviral vector prolonged survival in an ALS mouse model. However the last two studies only increased survival of the SOD1G93A transgenic mice by approximately 30%.

More recently, Raoul et al. (2005) showed that intraspinal injection of a lentiviral vector producing RNAi-mediated silencing of SOD1 substantially reduced both onset and progression of the disease in SOD1G93A transgenic mice. In addition, Ralph et al. (2005)

similarly demonstrated, through RNAi and lentiviral vectors, a delay in onset of ALS symptoms in SOD1G93A transgenic mice by more than 100% and an extension in survival by nearly 80% of their normal life span. The improved survival observed in these latter studies is probably due to the direct approach of targeting the genetic factor underlying the disease.

### 1.7.3 HSV-based Viral Vectors and Gene Therapy

This section will only briefly refer to herpes simplex virus type 1 (HSV-1) -based viral vectors for gene delivery in the central nervous system and highlight its potential as a means of efficient gene delivery to the CNS for the therapy of ALS and other neurodegenerative diseases. HSV-based vectors were used in this study (Chapters 4 and 5) as a highly efficient *in vitro* mechanism of gene delivery to cells, originally being developed for purposes of gene therapy. Information on the vectors utilised here will be provided (see also Introduction of Chapter 4) following an overview of HSV-1 and gene therapy.

#### 1.7.3.1 Basic HSV-1 biology and the rationale behind the development of HSV-1 vectors

The optimum HSV viral vector should not only deliver the gene of interest efficiently but also drive its long term expression and have no cytopathic effects; it should also be incapable of replication and unable to illicit an immune response and should not interfere with host cell's normal cellular functions. Herpes simplex virus-1 (HSV-1) based vectors are attractive for use in gene delivery as HSV-1 can infect a wide-range of dividing or non-dividing cells (i.e. virtually any human cell). In neurons the virus enters a lifelong latent state where it remains latent and episomal. The virus has evolved the ability of retrograde transport and can spread from the site of original infection to the site of latency in the spinal ganglia (Cook and Stevens, 1973). In addition, during latency, RNA species called the latency-associated transcripts (LATs) are generated by transcription of the long repeat region of the viral genome.

As reviewed by Latchman and Coffin (2001), HSV-1 infection of permissive cells results in the expression of more than 80 viral genes in a well coordinated manner of immediate early (IE), early (E), and late (L) gene products (Lokensgard et al., 1994) with subsequent cell lysis and release of progeny virions. Activation of IE genes occurs through the interaction of the tegument protein, vmw65 (also called virion protein 16, VP16 or a-transinducing factor, a-TIF) with cellular factors which then transactivate the promoters of the immediate early (IE) genes and as a result initiate an efficient lytic infection (Batterson et al. 1983). The initial expression of IE genes is enhanced by the tegument protein, vmw65 which enters the nucleus with the viral genome and works in cohort with the cellular factor octamer binding protein-1 (Oct-1) to bind to a consensus sequence in the IE gene promoter enhancers. Vmw65 cannot bind DNA directly and relies on the cellular POU domain protein Oct-1 and one other cellular factor known as host cell factor (HCF) in order to form a multicomponent complex on the TAATGARAT (where R is a purine) motifs which are present in all the IE gene promoters (Gaffney et al., 1985). Five virally encoded IE genes exist, they are: ICP0, ICP4, ICP22, ICP27, and ICP47.

In the course of a productive infection in cultured cells, HSV-1 gene expression takes place in a tightly regulated cascade which allows for the controlled expression of three classes of genes: the immediate early (IE) or "α" genes, the early (E) or "β" genes and the late (L) genes or "γ" genes (Honess and Roizman 1974). The "α" gene products, infected cell polypeptides (ICP) ICP0, ICP4, ICP22 and ICP27 all have regulatory functions, and they act together to regulate the expression of all classes of viral genes (Roizman and Sears, 1996). The "β" genes which are expressed next encode many of the proteins involved in the synthesis of viral DNA (Honess and Roizman 1974) and the late (L) genes or "γ" genes mainly encode the virion components (Batterson and Roizman 1983). With the exception of ICP47 (which is also an "α" gene product), the IE proteins have regulatory effects on E and L gene expression and are therefore responsible for co-ordinating a well-ordered temporal cascade of viral gene expression and viral replication.

Although it does not bind to DNA, ICP0 is an activator of E and L genes and therefore of viral replication. It is not essential for infection but its deletion significantly impairs viral replication. ICP4 encodes a nuclear phosphoprotein that is not only essential

for viral growth but also controls the expression of E and L genes through both transcriptional and posttranscriptional mechanisms (DeLuca and Schaffer 1985). In addition, ICP4 also negatively regulates IE gene expression and is therefore an essential regulatory IE gene and is the major transcriptional regulator of HSV-1 (DeLuca et al. 1985). ICP27 is required for the prevention of apoptosis in infected human cells. ICP27 also contributes to the shutoff of host protein synthesis observed during lytic infection, by means of impairment of host cell pre-mRNA splicing (Hardy and Sandri-Goldin 1994). ICP22 is not essential for viral growth but has been shown to promote efficient late gene expression in a cell type dependent manner (Sears et al. 1985). ICP47 is not essential for viral replication and does not play a part in IE gene expression. However, it has been shown that ICP47, which is a cytosolic protein, binds to the transporter associated with antigen processing (TAP) and as a result retains MHC class I molecules in the endoplasmic reticulum, and blocks peptide translocation. This consequently inhibits antigen presentation via the MHC class I pathway and is thought to be a mechanism through which the virus escapes host immunity (Hill et al. 1995).

Following IE gene expression, the next genes to be transcribed are the E genes. The E gene products include enzymes, which are essential for viral DNA replication. Synthesis of viral DNA takes place via a rolling circle mechanism that produces head-to-tail concatemers of the HSV-1 genome (Jacob et al. 1979). L gene expression is triggered by ICP4 and ICP27 and only takes place after viral DNA synthesis. L gene products include structural proteins of the capsid, tegument and envelope, which allow assembly of the virus. The viral DNA concatemers are cleaved into genome length units and packaged into the capsids within six hours of infection. The newly formed capsids covered with tegument proteins bud out of the nucleus, thereby acquiring the glycoprotein envelope and are transported to the cell surface via the endoplasmic reticulum. The lytic life cycle is rapid and results in cell death within ten hours of infection (Fink et al 1996).

Deletion of the major viral transactivator protein ICP4 dramatically reduces the expression of the ~80 HSV-1 genes. However, even though the majority of the virus genome is not transcribed, mutants incorporating the ICP4 deletion are still cytotoxic (Marshall et al., 2000). The majority of this toxicity is due to the products of the other regulatory IE

genes, ICP0, ICP22, and ICP27 (Marshall et al., 2000; McFarlane et al., 1992; Palella et al., 1988) and is further reduced when these other regulatory IE genes are inactivated together with ICP4. Thus, an optimal HSV vector should not express significant amounts of any of the regulatory IE genes.

### 1.7.3.2 Viral vectors used in this study

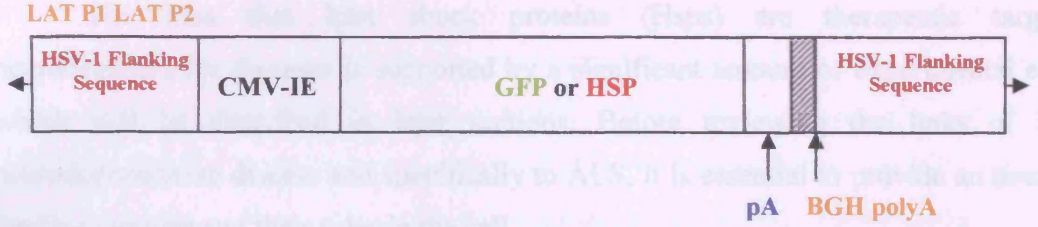
The viruses utilised in this study were made by Dr. Marcus Wagstaff and Dr Rob Coffin. The Hsp and HSF-1 viruses created by Dr Marcus Wagstaff have proved to be very useful in efficiently over-expressing Hsps or HSF-1 in neuronal (Wagstaff et al., 1999; Wagstaff et al., 1998; Zourlidou et al; 2004) and non-neuronal cells (Brar et al., 1999; Jamshidi et al., 2004). The SOD1 viruses created by Dr Rob Coffin have been used in this study to deliver wt-SOD1 and G93R-SOD1 mutant to neuronal cells (Patel et al., 2005). The recombinant vectors 17+pR19 and 17+pR20.5 were designed so that they can express high levels of the transgene (Hsps or reporter genes) during latency (see also Introductions of Chapter 4 & Chapter 5).

The Hsp and HSF-1 transgenes were driven by the human cytomegalovirus immediate-early (CMV-IE) promoter that was inserted immediately downstream of the latency associated transcript promoter 2 (LAT P2); (figure 1.5a). This expression construct was introduced into the non-essential LAT region of the IE2 deleted mutant HSV-1 DNA. The CMV-IE promoter drives the transcription of the transgene as well as the bovine growth hormone polyadenylation sequence. The use of the LAT P1 and LAT P2 promoters with the CMV-IE promoter is thought to facilitate expression throughout viral latency in neurons.

For the SOD viruses the vector also contained green fluorescent protein (GFP) reporter gene whose expression was driven by CMV promoter with the SOD1 gene expression driven by the RSV promoter, with the latent associated transcript LAT P2 region (from HSV-1) between the two promoters to gain long-term expression of both transgenes (figure 1.5b). This virus lacks the ICP27 gene, is deleted for the endogenous LAT P2 regions thereby preventing recombination with the inserted LAT P2-containing expression cassette pR20.5 outside the LAT region. The pR20.5 cassette consists of a central LAT P2

element flanked by two heterologous promoters (CMV-IE and RSV) arranged in a back-to-back orientation, allowing simultaneous expression of either wt-SOD1 or G93R-SOD1 mutant (under the Rous Sarcoma Virus - RSV- promoter) and GFP (under CMV-IE promoter) and enabling long term expression of both transgenes. This cassette was inserted into a plasmid to enable insertional inactivation of the gene encoding virion host shut off protein (vhs), which has been shown to play a role in pathogenesis and latency (Strelow and Leib, 1995) and further disabled the HSV virus.

**(a)**




**(b)**

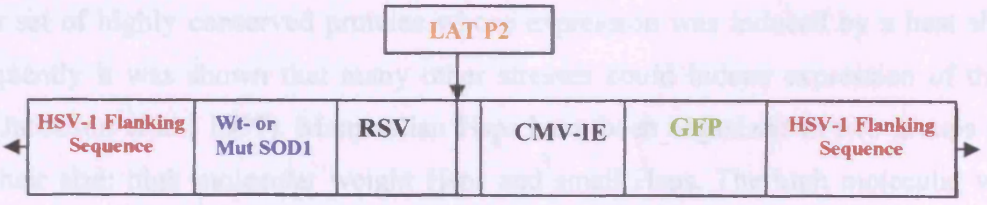



**Figure 1.5** **(a)** The pR19 cassette utilising the 17+ virus construct **(b)** The pR20.5 cassette utilising the 17+ virus constructs.

Figure 1.5 shows the expression cassettes, which were inserted into the non-essential 2 kb LAT region of the HSV-1 genome of an IE2-deleted mutant HSV-1 strain (IE2 is the essential gene that encodes ICP27; HSV-1 strain 17+; GenBank™ accession number

HE1CG) (Wagstaff et al., 1999). The IE genes ICP4 and ICP27 are essential for viral replication. Therefore ICP27 was deleted in these virus types. These viruses were designed to produce a less cytopathic, latent, non-replicating viral mutant, which was tested and found to be suitable for gene delivery (Wagstaff et al., 1999; Kalwy et al., 2003). These viruses were grown on B130/2 cells which complement for the deleted ICP27 (Howard et al., 1998).

## 1.8 Heat Shock Proteins

### 1.8.1 Overview

The idea that heat shock proteins (Hsps) are therapeutic targets for neurodegenerative diseases is supported by a significant amount of experimental evidence, which will be described in later sections. Before reviewing the links of Hsps to neurodegenerative disease and specifically to ALS, it is essential to provide an overview of the Hsp families and their roles in the cell.

Stress or heat shock proteins (Hsps) were first discovered in 1962 (Ritossa, 1962) as a set of highly conserved proteins whose expression was induced by a heat shock. Subsequently it was shown that many other stresses could induce expression of these proteins (Jacobson et al., 1997). Mammalian Hsps have been organized in two groups according to their size: high molecular weight Hsps and small Hsps. The high molecular weight group includes three major families: Hsp90, Hsp70, and Hsp60. Some of them are expressed constitutively while the expression of others is stimulated by stressful conditions. In addition, these proteins can undergo relocation to different sub-cellular compartments. High molecular weight Hsps, such as Hsp70, are ATP-dependent chaperones and need co-chaperones to modulate their conformation and ATP binding. In contrast, small Hsps, for instance Hsp27, are ATP-independent chaperones (Jacobson et al., 1997). The term molecular chaperone is given to a functionally related set of proteins divided into several classes or families according to their molecular weight. A cell may express multiple members of the same chaperone family; for example, the yeast *S. cerevisiae* produces 14 different versions of the chaperone Hsp70 (Craig et al., 1999). Proteins from the same class

of molecular chaperones frequently exhibit significant amounts of sequence homology and are structurally and functionally related, while there are hardly any homology exists between chaperones from different families. However, in spite of this diversity, most molecular chaperones share common functional features.

Hsps have a cytoprotective function through which they can enable cells to adapt to gradual changes in their environment and to survive in otherwise lethal conditions. The different pathways through which Hsps modulate cell death are only now beginning to be understood.

### 1.8.2 Heat shock proteins function as chaperones

The Hsp70 family of stress proteins represents the most conserved and best studied class of Hsps. Human cells possess several Hsp70 family members including stress inducible Hsp70, constitutively expressed Hsc70, mitochondrial Hsp75, and Grp78, localized in the endoplasmic reticulum (Jaatela et al., 1999). Under normal conditions, Hsp70 proteins act as ATP-dependent molecular chaperones by assisting in the folding of newly synthesized polypeptides as well as the assembly of multiprotein complexes, in addition to the transport of proteins across cellular membranes (Murakami et al., 1988; Beckmann et al., 1990; Shi et al., 1992). Under conditions of stress, the synthesis of inducible Hsp70 boosts the ability of cells to deal with increased concentrations of unfolded or denatured proteins (Nollen et al., 1999).

Principal members of the Hsp90 family comprise the ATP-dependent chaperones Hsp90α, Hsp90β, and Grp94 (Csermely et al., 1998). The two Hsp90 isoforms are constitutively expressed in eukaryotic cells and are essential for their viability. These proteins constitute 1-2% of cytosolic proteins and can accumulate further in response to stress. Hsp90 proteins have been shown to associate with several signalling proteins, including ligand-dependent transcription factors such as steroid receptor (Nathan et al., 1995), ligand-independent transcription factors such as MyoD (Shaknovich et al., 1992), tyrosine kinases such as v-Src (Hartson et al., 1994), and serine/threonine kinases such as

Raf-1 (Wartmann et al., 1994); the main chaperone role of these proteins is the promotion of the conformational maturation of these transcription factors and signal transducing kinases.

Mammalian Hsp60, also known as chaperonin, is a constitutive protein found mainly within the mitochondrial matrix, although 15-20% of the protein is located in the cytosol. Hsp60 plays a part in the folding of mitochondrial proteins and assists in the proteolytic degradation of misfolded or denatured proteins in an ATP-dependent manner. The chaperone function of Hsp60 is regulated by its co-factor Hsp10, which binds to Hsp60 and regulates substrate binding and ATPase activity. In the presence of ADP, two molecules of Hsp10 bind to one Hsp60 molecule. Hsp60 and Hsp10 do not always operate as a single functional unit, as only newly imported proteins are severely affected by inactivation of Hsp10 (Bukau et al., 1998).

Proteins of the small Hsp family, that include Hsp27 and the α-crystallins, range in size from 15 to 50kDa and share sequence homologies as well as biochemical properties such as phosphorylation and oligomerization. For instance, Hsp27, a protein abundantly expressed in several cell types and tissues at specific stages of differentiation and development, can form oligomers up to 1000kDa in size. These powerful ATP-independent chaperones defend the cell against protein aggregation (Ehrnsperger et al., 1997). The affinity of small Hsps for the proteins to be chaperoned is most probably modulated through their phosphorylation and oligomerization status. The dimer of Hsp27 appears to be the building block for the multimeric complexes. Hsp27 oligomerization is a dynamic process, which is dependent on the phosphorylation status of the protein and exposure to stress (Garrido et al., 2002). Hsp27 can be phosphorylated at three serine residues with oligomerization being enhanced through its dephosphorylation. This phosphorylation is a reversible process catalyzed by the MAPKAP kinases 2 and 3 in response to a range of stresses, including differentiating agents, mitogens, inflammatory cytokines such as TNFα and IL-1β, hydrogen peroxide, and other oxidants.

Most Hsps are involved in the proper folding and/or elimination of misfolded proteins, thereby contributing to cell survival. Some of the important house-keeping functions ascribed to the molecular chaperones include: (1) the import of proteins into

cellular compartments; (2) the folding of proteins in the cytosol, endoplasmic reticulum, and mitochondria; (3) the degradation of unstable proteins; (4) dissolution of protein complexes; (5) prevention of protein aggregation; (6) the control of regulatory proteins; as well as (7) the refolding of misfolded proteins (Bukau et al., 1998). The accumulation of abnormally folded proteins in the nucleus or the cytosol that comes about as a consequence of stress, such as increased temperatures, free oxygen radicals, heavy metals, and even antibiotics (Sherman et al., 2001), results in the formation of aggregates that disturb normal cellular function and as a result trigger cell death (Sherman et al., 2001). Such a mechanism appears to be involved in the pathogenesis of lesions that are characteristic of many neurodegenerative disorders.

The factors that define the specificity of the chaperone activity are the structure of the chaperone as well as the size and localization of the protein to be chaperoned (Table 1.2). Recently, it has been recognized that Hsps also regulate apoptosis with Hsp27 and Hsp70 possessing antiapoptotic properties, while Hsp60 and Hsp10 are thought to possess both proapoptotic or anti-apoptotic properties depending on sub-cellular localisation - mitochondrial (Samali et al., 1999; Xanthoudakis et al., 1999) or cytoplasmic (Lin et al., 2001; Kirchhoff et al., 2002) respectively. Hsps are thought to function at multiple points in the apoptotic signalling pathway (Fig. 1.6).

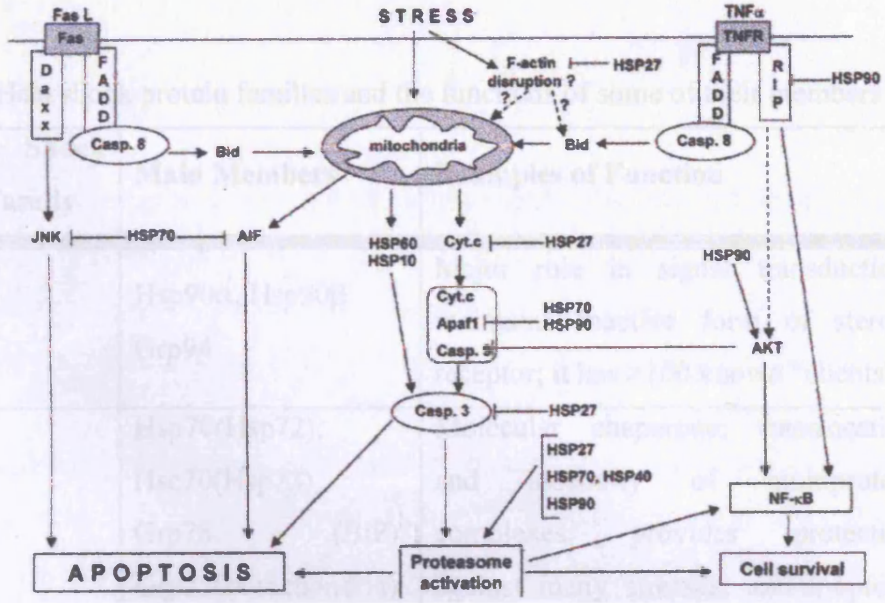



**Figure 1.6** Diagram illustrating the regulation of mitochondrial cell death pathways by Hsps. Hsp27 binds to cytochrome c (Bruey et al., 2000); Hsp70 and Hsp90 bind to Apaf-1 (Beere et al., 2000; Pandey et al., 2000; Saleh et al., 2000) in all cases leading to inhibition of caspase activation. Additional negative effect of Hsp27 and Hsp70 on events both further upstream and downstream have been described (Jaattela et al., 1998; Paul et al., 2002; Pandey et al., 2000). Hsp60 and Hsp10 possess the ability to directly promote the proteolytic maturation of caspase-3 (Samali et al., 1999; Xanthoudakis et al., 1999). Hsp90 interacts with RIP-1 kinase and Akt and thereby promotes NF-κB-mediated inhibition of apoptosis (Lewis et al., 2000; Sato et al., 2000). Induction of caspase activation triggers proteasome activation (Dallaporta et al., 2000). Hsps facilitate the degradation of proteins via the ubiquitin/proteasome system (Mathew et al., 2001). Figure adapted from Parcellier et al., (2003).

Hsps are grouped into families according to their molecular weight however there is some confusion that exists about their nomenclature. Table 1.2 provides a list of the major Hsp families; references are not included in the table but are cited in the text when an Hsp is mentioned.

**Table 1.2** Heat shock protein families and the functions of some of their members

| Heat Shock Protein Family | Main Members | Examples of Function |
|---|---|---|
| Hsp90 | Hsp90α, Hsp90β<br>Grp94 | Major role in signal transduction; maintains inactive form of steroid receptor; it has >100 known "clients" |
| Hsp70 | Hsp70(Hsp72), Hsc70(Hsp73), Grp78 (BiP), Grp75(mitochondria), Hsx70 (primates only) | Molecular chaperone; translocation and assembly of multiprotein complexes; provides protection against many stresses; anti-apoptotic actions; degradation of substrates via the proteasome |
| Hsp60 | Hsp60 (mitochondria), GroEL (prokaryotic) | Folds "molten globule" proteins or domains; mitochondrial protein folding |
| Hsp56 | Hsp56 (FKBP56) | Protein folding; maintains inactive form of steroid receptor; a co-factor of Hsp90 |
| Hsp40 | Hdj1 (Hsp40), Hdj2(Hsdj1), Hsc40, Csp, Tpr2, auxilin, etc | Protein folding; stimulates Hsp70 ATPase activity in PD and polyglutamine disease inclusions |
| Hsp32 | Hsp32 (HO1) | Antioxidant |
| Hsp27 | Hsp27, Hsp25, Hsp22,Hsp26, αA-crystallin, αB-crystallin | Actin binding; protein folding; anti-apoptotic functions; degradation of certain substrates via the proteasome; provides protection against stresses |
| Others | Ubiquitin<br>Hsp10 | UPS-Protein degradation (ubiquitin)<br>A regulator and co-factor of Hsp60 |

### 1.8.3 Hsps as anti-apoptotic molecules

For some time now it has been known that cells induced to accumulate Hsps subsequently become more tolerant to cytotoxic insults, a phenomenon termed "thermotolerance" (Parsell et al., 1993; Li et al., 1980; Li et al., 1982). This increased cellular survival is linked to the expression of Hsps and their ability to prevent cell death.

The recent discovery of relationship between Hsp expression and increased cell survival, has pointed to Hsps as playing a crucial role in the regulation of the apoptotic cell machinery. Investigations have confirmed that Hsp27 and Hsp72 increase cell survival in response to apoptotic stimuli (Mehlen et al., 1996; Mosser et al., 1992; Samali et al., 1996). Therefore it is apparent that the protective effect of Hsps during thermotolerance is due, to some extent, to the ability of these proteins to inhibit apoptosis (Mosser et al., 1992). A large amount of attention has focused on elucidation of the molecular mechanism by which Hsps mediate their anti-apoptotic abilities, due to their potential role as key death determinants within cells. Hsp72 has been reported to inhibit apoptosis via a direct interaction with Apaf-1, thus preventing the docking and subsequent activation of pro-caspase-9 (Beere et al., 2000; Saleh et al., 2000). Furthermore, Hsp90 negatively regulates caspase activity by inhibiting the cytochrome c-mediated Apaf-1 oligomerization (Pandey et al., 2000). In accordance with these observations interactions between Hsp27 and key components necessary for the activation of caspases have also been observed.

The following sections discuss Hsp70 and Hsp27 which are the major Hsps used in this study and their roles in stress, in cell death pathways, in proteolytic degradation by the proteasome and finally in neurodegeneration and ALS in particular.

### 1.8.4 The Hsp70 System

The Hsp70 proteins make up the central part of a ubiquitous chaperone system that is present in most compartments of eukaryotic cells, in eubacteria, as well as in many archaea. The most extensively studied representative of this chaperone group is the DnaK protein, from the bacterium *E. coli*; DnaK is the Hsp70 of *E. coli*. The Hsp70 proteins are involved

in a wide variety of cellular processes, such as protein folding as well as in the degradation of unstable proteins. In these processes, the common function of Hsp70 appears to be that of binding short hydrophobic segments in partially folded polypeptides, thus preventing aggregation and arresting the folding process (Craig et al., 1999; Flynn et al., 1991).

*In vivo*, DnaK and many other Hsp70 chaperones interact with two classes of partner proteins, the Hsp40 and the GrpE proteins, which regulate critical steps of its functional cycle. Moreover, in the past years, additional partner proteins have been identified, particularly in eukaryotic cells, and a number of them link Hsp70 to other chaperone systems.

### 1.8.4.1 Structural and Functional Properties of Hsp70

Hsp70 is made up of two functional domains, one is an N-terminal ATPase domain, and the second a smaller C-terminal peptide-binding domain. Both the crystal structures of the ATPase domain and the peptide-binding domain of DnaK have been determined (Flaherty et al., 1990; Harrison et al., 1997; Zhu et al., 1996). The Hsp70 ATPase domain consists of two subdomains separated by a cleft containing the nucleotide-binding site (Flaherty et al., 1991). The peptide-binding properties of the C-terminal domain are determined by the nature of the bound nucleotide. In the ATP state, although with low affinity, there is a rapid binding and dissociation of the peptide substrates (Schmid et al., 1994). However, the rates of peptide binding and dissociation decrease by more than two orders of magnitude when there is no nucleotide or ADP bound to the N-terminal domain, whereas the affinity increases significantly. Therefore, ATP hydrolysis acts as a molecular switch for Hsp70 between the two states, which are characterized by high dynamics/ low affinity or low dynamics/high affinity. As yet, the full-length structure of Hsp70 has not been determined, hence, we have no direct information on how communication between nucleotide binding and peptide binding takes place at a molecular level. However, insight into how this communication might occur may be provided by the X-ray crystal structure of the peptide-binding domain of DnaK co-crystallized with a heptapeptide bound to its active site (Zhu et al., 1996).

### 1.8.4.2 Hsp70: A Potent Anti-apoptotic Protein

Hsp70 has been shown to inhibit apoptosis and as a result increase the survival of cells exposed to a wide range of lethal stimuli (Jaattela et al., 1992; Mosser et al., 1997). Hsp70 overexpression protects cells from stress-induced apoptosis, both upstream and downstream of the caspase cascade activation (Fig. 1.6). In addition, some preliminary data suggests that Hsp70 could protect the cells from energy deprivation and/or ATP depletion associated with cell death (Wong et al., 1998).

Elevated levels of Hsp70, achieved through either transient or stable transfections, not only reduce or block caspase activation but also suppress mitochondrial damage and nuclear fragmentation (Buzzard et al., 1998). Further support of these findings were provided by Li et al. (Li et al., 2000) who found Hsp70 inhibited apoptosis downstream of cytochrome c release and upstream of caspase-3 activation. This antiapoptotic effect is exerted through the Hsp70-mediated modulation of the apoptosome. Hsp70 has been demonstrated to directly bind APAF-1, and as a result prevent the recruitment of procaspase-9 to the apoptosome (Saleh et al., 2000; Beere et al., 2000). The ATPase domain of Hsp70 is thought to be essential for this interaction (Saleh et al., 2000; Mosser et al., 2000).

Interestingly, Hsp70 may prevent cell death in conditions during which caspase activation does not take place either as a result of addition of exogenous caspase inhibitors (Creagh et al., 2000) or after inactivation of the apaf-1 gene (Ravagnan et al., 2001). In addition, it has been shown that cells deleted of apaf1 are protected from apoptosis induced by serum withdrawal by over-expression of Hsp70 (Ravagnan et al., 2001); indicating that Apaf-1 is not the sole target of the antiapoptotic action of Hsp70. Hsp70 appears to directly bind AIF (apoptosis inducing factor) and prevent AIF-induced chromatin condensation. Hsp70 has been found to neutralize the apoptogenic effects of AIF not only *in-vitro*, but also in cells microinjected with recombinant Hsp70 and/or AIF protein, in addition to cells transiently transfected with AIF cDNA. Hsp70 has been shown to inhibit apoptosis induced by overexpression of full-length AIF, which has to transit mitochondria to become apoptogenic (Susin et al., 1999; Loeffler et al., 2001), as well as AIF deficient for the

mitochondrial localization sequence (AIFA1-100). It appears endogenous levels of Hsp70 are sufficiently high enough to control AIF-mediated apoptosis as down-regulation of Hsp70 through use of antisense construct, sensitizes the cells to serum withdrawal and AIF (Ravagnan et al., 2001).

In addition, Hsp70 has also been shown to rescue cells from a later stage of apoptosis than any known survival-enhancing drug or protein. In TNF-induced apoptosis, Hsp70 did not prevent the activation of caspase-3 but exerted effects further downstream preventing morphological changes characteristic of dying cells (Jaattela et al., 1998). Hsp70 is also thought to act at earlier stages of the apoptotic pathway, for instance by preventing JNK activation (Meriin et al., 1999). Park et al. (2001) have recently shown that Hsp70 binds to and acts as a natural inhibitory protein of c-Jun N-terminal Kinase (JNK1) and that the binding is independent of the ATPase domain of Hsp70 (Mosser et al., 2000). However, JNK inhibition accomplished through this binding is not sufficient to prevent apoptosis (Mosser et al., 2000), putting a question mark over the role of JNK itself in apoptosis. In addition, Hsp70 has also been shown to associate with the pro-apoptotic proteins p53 and c-myc (Jolly et al., 2000). However, the functional impact of these interactions in the "Hsp70 survival effects" remains unknown (Jolly et al., 2000). Another apoptosis regulatory protein, which interacts with Hsp70, is Bag-1, reported to function as a co-chaperone of Hsp70, and simultaneously regulating the activities of proteins such as Bcl-2 and Raf-1. Hsp70/Bag-l has been shown to regulate Raf-1/ERK kinase and cell growth in response to stress (Song et al., 2001).

The data discussed above shows that Hsp70 inhibits apoptosis and these properties may be attributed to the neutralizing interactions of Hsp70 with several proapoptotic effectors including Apaf-1, AIF and possibly signalling molecules such as JNK-1, p53, or c-myc. Presently, it is not clear whether these neutralizing interactions synergize in some way to determine the broad cytoprotective effect of Hsp70.

In addition to the roles of Hsp70 described so far, there is also strong evidence for a cytoprotective role for Hsp70 under different stresses (mainly heat and hypoxia and ischemia/reperfusion), as demonstrated by various *in vitro* and *in vivo* studies (Kiang and

Tsokos, 1998). The present study deals with the inducible Hsp70, the over-expression of which is known to protect several systems under conditions of stress.

### 1.8.4.3 Protective properties of Hsp70 in neuronal systems *in vitro*

The following studies provide some examples of *in vitro* protection achieved through over-expression of Hsps in a variety of different cell types. Kabakov et al. (2003) reported protection from delayed apoptosis following over-expression of Hsp70 in human endothelial cells during post hypoxic reoxygenation. Brar et al. (1999) demonstrated in primary cardiomyocytes, that over-expression of Hsp70 via HSV-based vector protects against apoptosis-inducing stimuli as well as against thermal and hypoxic stress. In addition, studies on cardiomyocyte-derived cell lines or primary cells either transiently or stably over-expressing Hsp70 also indicated a strong protective effect of Hsp70 under both thermal and ischemic stresses (reviewed by Latchman, 2001). In cell culture models over-expression of inducible Hsp70 protects from ischemia (Papadopoulos et al., 1996; Xu and Giffard, 1997; Kiang and Tsokos, 1998). In addition, Hsp70 over-expression through HSV-based viral vectors protects dorsal root ganglion neurons from thermal and ischemic stresses but was found to have no protective effect against apoptosis (Wagstaff et al., 1999).

### 1.8.4.4 Protective properties of Hsp70 in neuronal systems *in vivo*

The following studies provide some examples of *in vivo* protection achieved in different cell types through over-expression of Hsp70. Transgenic mice over-expressing Hsp70 demonstrated less infarction than the wt-littermates, following transient focal ischemia, transient global ischemia, or kainic acid-induced seizures; in addition Hsp70 over-expression reduced the number of apoptotic cells (Tsuchiya et al., 2003). Over-expression of inducible Hsp70 by either viral or transgenic expression has been shown to provide protection against cerebral ischemia in animal models of stroke (Plumier et al., 1997; Rajdev et al., 2000; Yenari et al., 1998 & 1999), but as yet the mechanisms of this neuroprotection are not fully understood. Interestingly, Hsp70 was recently shown to reduce protein aggregation in a model of global ischemia and its induction with geldanamycin blocked apoptotic astrocyte death induced by glucose deprivation (Giffard et al., 2004).

Overall, it is clear Hsp70 and members of the Hsp70 family have many important and diverse roles. However, the principal function of these proteins is to prevent the misfolding of denatured proteins. Under conditions of stress, increasing the levels of Hsp70 may prevent aggregation and misfolding of denatured proteins and assist in either their correct folding or their degradation by the proteasome. The potential role of the proteasome in neurodegeneration is discussed later in a separate section. Additional studies on Hsp70 are needed due to its clinical importance and its potential as a therapeutic agent.

### 1.8.4.5 Hsp70 and co-factor roles on protein degradation by the proteasome

The ability to facilitate the degradation of intracellular proteins by the ubiquitin/proteasome system is another protective function recently proposed for Hsps. The ubiquitinylation system marks proteins for degradation by the 26S proteasome, a multicatalytic protease consisting of a catalytic 20S and a regulatory 19S subunit. One of the key signals that allow protein recognition is misfolding. A cooperation has been reported between the ATP-dependent molecular chaperones Hsp90 and Hsp70 and the ubiquitinylation machinery, for instance, it is thought the proteins could encourage the degradation of proteins such as the endoplasmic reticulum-associated apoprotein B by the ubiquitin-proteasome (Gusarova et al., 2001), but as yet, the molecular mechanisms involved remain largely unknown.

The co-chaperone CHIP (C-terminus of Hsc70-interacting protein) is an ubiquitin ligase, which on interaction with Hsp70, efficiently ubiquitinylates captured unfolded proteins, and subsequently associates with the 19S subunit of the proteasome (Council et al., 2001; Murata et al., 2001; Meacham et al., 2001). The ubiquitin-like domain containing protein BAG-1, which is another co-chaperone for Hsp70 and Hsc70, also binds to the proteolytic machinery in an ATP-dependent manner, supplying an additional link between the Hsc70/ Hsp70 chaperone system and the 26S proteasome (Luders et al., 2000). BAG-1 and CHIP cooperate to shift the activity of the chaperone systems from one of protein folding to that of protein degradation (Demand et al., 2001).

The role of Hsps in the proteosomal degradation of unfolded substrates demonstrates a degree of specificity, in that Hsc70 for instance, is necessary for the degradation of actin, α-crystallin, glyceraldehyde-3-phosphate dehydrogenase, β-lactalbumin, and histone H2A, but is not required for the degradation of bovine serum albumin, lysozyme, and oxidized RNase A (Bercovich et al., 1997). In the same way by interacting with CHIP, Hsp70, and Hsp90 specifically favour the degradation of the Parkin protein, CFTR (cystic fibrosis transmembrane-conductance regulator) and the glucocorticoid receptor (Meacham et al., 2001).

## 1.8.5 The Small Heat Shock Protein Family

### 1.8.5.1 Overview of Hsp27 and the small Hsp family

Hsp27 is a member of the small heat shock proteins (sHsps), a family of abundant and ubiquitous stress proteins, which are detectable in virtually all organisms from prokaryotes to mammals (Arrigo et al., 1994). To date, nine different members of the family of sHsps have been identified, varying in size from 15 to 30 kDa: Hsp27, p20, HspB3, MKBP/HspB2, HspB8, HspB9, cvHsp, α-A crystallin and α-B crystallin (Arrigo et al., 1987; Boelens et al., 1998; Ingolia et al., 1982; Iwaki et al., 1997; Kappe et al., 2001; Kato et al., 1994; Klemenz et al., 1991; Krief et al., 1999). Although low amino acid homology is shared between members of this family, they are grouped together because of their similar structural and functional properties, with all sHsps containing a conserved core region that was identified first within the crystallin proteins of the vertebrate eye (De Jong et al., 1993). Termed the crystallin box, this domain consists of 80-100 amino acids in the C-terminus of the protein and has an IgG-like fold, which is proceeded by a short more freely flexing C-terminal extension. In contrast, the N-terminus of sHsps is a lot more variable in both sequence and length (De Jong et al., 1998) and contains the WDPF motif, involved in oligomerization of the protein (Bova et al., 2000).

Hsp27 levels are generally low within unstressed cells and it exists predominantly as a large oligomeric unit of up to 800 kDa, frequently comprised of six tetrameric complexes

of the protein. The size of the oligomeric unit depends on several physical and chemical parameters, which include temperature, pH, ionic strength and the degree of phosphorylation of the individual monomers. An increase in the level of expression of Hsp27 during the stress response is preceded by a phosphorylation-induced reorganization of the multimeric status of the protein. Phosphorylation takes place on three different serine residues, Ser-15, Ser-78 and Ser-82 and results in the redistribution of the large oligomer into smaller tetrameric units (Rouse et al., 1994; Lavoie et al., 1995; Zantema et al., 1992). Phosphorylation of Hsp27 is catalysed by MAPKAP kinases 2 and 3 (Landry et al., 1992; Ludwig et al., 1996; Stokoe et al., 1992), which in turn are activated by the phosphorylation of p38 MAP kinase (Freshney et al., 1994). Recent evidence suggests that the delta isoform of protein kinase C may also phosphorylate Hsp27, however, this only seems to occur in a stimulus-dependent manner (Kato et al., 2001), such as with treatment with phorbol esters. Increased phosphorylation of Hsp27 is detectable, after exposure to stress, within several minutes with a subsequent increase in the expression levels of the protein occurring within several hours (Landry et al., 1991). The induction of Hsp27 is generally transient and the protein returns to basal levels following removal of the stress. Interestingly, at specific stages during development and cell differentiation, increased expression of Hsp27 is transiently induced and occurs alongside the differentiation-mediated decrease of cellular proliferation (Pauli et al., 1989). Experiments in mouse embryonic stem cells suggest that aberration of this accumulation is enough to abort the differentiation process resulting in the death of the cells (Mehlen et al., 1997).

The complexes formed by the sHsps with non-native proteins *in vitro* are extremely stable (Lee et al., 1997; Ehrnsperger et al., 1997 & 1998; Haslbeck et al., 1999). Even though the non-native protein is not spontaneously released, these complexes are not dead-end traps for the unfolded protein. It has been demonstrated, as proof of principle, that a bound enzyme can be shifted back to its native state by addition of a specific ligand that stabilizes the functional conformation of the protein (Ehrnsperger et al., 1997). However, up to now and in contrast to other chaperones, no active-release mechanism has been detected. Interestingly, in a number of organisms the expression of a sHsp and an Hsp70 chaperone is genetically linked (Michelini et al., 1999). This along with the finding that the *in vivo* overexpression of Hsp70 has a beneficial effect on the clearance of aggregates suggests that

sHsps may operate together with other ATP-dependent members of the chaperone family (Kampinga et al., 1994). *In vitro* experiments, in which Hsp70 was added to preformed sHsp/substrate complexes has provided biochemical proof for this co-operation. Hsp70 was able to promote folding of the protein to its native state in the presence of ATP (Ehrnsperger et al., 1997). Independently, experiments which screened cell lysates for factors that promote the refolding of sHsp-bound proteins (Lee et al., 1997 & 2000) also identified Hsp70 as the significant player in the reactivation of proteins rescued from aggregation by sHsps.

It appears sHsps function by binding non-native proteins once large quantities of unfolded proteins are formed, as can happen as a result of stress conditions or overexpression of proteins. This binding prevents the formation of large aggregates and subsequently makes it easier for Hsp70 or other potential ATP-dependent chaperone systems to refold the protein. This cooperation of different components of the cellular chaperone machinery therefore enables the separation in both space and time of two key properties of molecular chaperones, binding and folding.

### 1.8.5.2 Hsp27 and its mechanism of cytoprotection

A range of different roles for Hsp27 during cellular stress have been put forward to account for the cytoprotective effects observed with increased expression of this protein such as its role as a molecular chaperone, direct interference with the mechanisms of caspase activation, modulation of oxidative stress and regulation of the cytoskeleton.

### 1.8.5.3 Hsp27 as a molecular chaperone

Like other members of the Hsp family, Hsp27 functions as a molecular chaperone helping to refold non-native proteins. By forming complexes with such proteins, it prevents their non-specific aggregation thereby allowing them to be subsequently restored to their native structure in co-operation with ATP-dependent chaperones such as Hsp70 (Beissinger et al., 1998; Ehrnsperger et al., 1997). Unlike most other Hsps, the chaperone function of sHsps occurs in an ATP-independent manner (Jakob et al., 1993) with the C-terminal

responsible for the molecular chaperone function (Muchowski et al., 1997). The intracellular accumulation of misfolded proteins can elicit a stress response resulting in increased Hsp expression (Ananthan et al., 1986). Excessive amounts of damaged proteins can form large aggregates, which act as a signal for the induction of apoptosis (Soldatenkov et al., 1997).

The increased expression of Hsp27, in stressed cells containing high levels of damaged proteins, assists in the repair or destruction of these proteins thereby promoting recovery of the cell. This ability of Hsp27 to assist the recovery of stress-induced protein denaturation may increase the cell survival rate by limiting the amounts of misfolded proteins that may potentially be responsible for triggering of apoptosis. For example, overexpression of Hsp27 enhances the rate of recovery from aggregation of nuclear protein (Kampinga et al., 1994). During the temperature-induced stress response, increased expression of Hsps is accompanied by a shut-off of general protein and mRNA synthesis (DiDomenico et al., 1982). Cuesta and co-workers (2000) have shown that Hsp27 can itself operate as an inhibitor of cellular protein synthesis. They showed that Hsp27 interacted *in vitro* with eIF4G, a cap-binding initiation factor necessary for translation of most cellular mRNAs. The eIF4G factor was prevented from initiating the start of translation as a result of this interaction (Cuesta et al., 2000). Since under stress conditions, protein synthesis could potentially result in their incorrect folding and accumulation of protein aggregates, this interaction could be a protective mechanism to further restrict stress related accumulation of misfolded proteins. Interestingly, Hsp27 expression has also been reported to promote recovery of RNA and protein synthesis following heat shock (Carper et al., 1997), which may provide the cell with a survival advantage.

Furthermore, the molecular chaperone function of Hsp27 is responsible for the regulation of apoptosis by means of its interaction with protein kinase B (Akt). Akt activation has been shown to inhibit apoptosis in a number of systems (Ahmed et al., 1997; Kaufmann-Zeh et al., 1997; Kennedy et al., 1997; Khwaja et al., 1997; Kulik et al., 1997). Akt is activated through an association with HSP27, during both heat and hydrogen peroxide-induced cell stress (Konishi et al., 1997). This Hsp27-mediated activation of Akt is likely to play a part in the increased resistance to apoptosis seen in cells expressing high levels of Hsp27 (Kaufmann-Zeh et al., 1997).

### 1.8.5.4 Inhibition of caspase activation and activity

Over recent years, evidence showing that Hsp27 can inhibit apoptosis by means of a direct inhibition of caspase activation has accumulated (Garrido et al., 1999; Samali et al., 2001). It is not implausible, that as a molecular chaperone Hsp27 could regulate the activation of caspases by way of an ability to interact with one or more components of the apoptosome complex/caspase-9 activation complex; the apoptosome is formed by interaction of cytochrome-c with Apaf-1, triggering the ATP-dependent oligomerization of Apaf-1 and it's binding to procaspase-9. So far, a number of different studies have provided evidence of interactions of Hsp27 with critical components of the apoptosome. Hsp27 negatively regulates the activation of pro-caspase-9 through an ability to interact with cytochrome c, which consequently prevents the correct formation/function of the apoptosome complex (Bruey et al., 2000; Concannon et al., 2001). In addition, it has also been shown that by interacting with the pro-caspase-3 molecule, Hsp27 can inhibit caspase-3 activity (Concannon et al., 2001; Pandey et al., 2000). Interestingly, even though Pandey et al. (Pandey et al., 2000) were unable to show a direct interaction between Hsp27 and cytochrome c they conclude that Hsp27 may play a role both upstream and downstream of cytochrome c release in a stimulus-dependent manner.

Recent evidence has demonstrated that the mitochondrial fraction of thermotolerant Jurkat cells contains a significant pool of Hsp27 (Samali et al., 2001). This is reminiscent of another endogenous anti-apoptotic protein Bcl-2, which functions to inhibit apoptosis by preventing cytochrome c release from the intermembrane space of mitochondria (Kluck et al., 1997; Yang et al., 1997). Therefore it is interesting to speculate that Hsp27 may operate in a fashion similar to Bcl-2. It has been observed that Hsp27 may protect against apoptotic stimuli by blocking cytochrome c release (Samali et al., 2001).

Exposure of cells rendered thermotolerant to an apoptotic stimulus did not result in loss of mitochondrial membrane potential ($\Delta\psi m$) and subsequent release of cytochrome c associated with non-thermotolerant cells (Samali et al., 2001). When a similar experiment was performed using cells transfected with antisense Hsp27, the loss of $\Delta\psi m$ and release of cytochrome c were not inhibited (Samali et al., 2001), therefore suggesting that in

thermotolerant cells Hsp27 is responsible for inhibition of these factors. On the other hand, Bruey et al. (Bruey et al., 2000) did not see an effect by Hsp27 on release of cytochrome c. One possible explanation for these conflicting observations may be the different model systems used in these experiments. Usually, when Hsp27 is transfected into cells its expression is under control of foreign viral gene promoters and not as is the case during heat shock, the transcription factor, HSF-1. Under conditions of stress, HSF-1 not only mediates the induction of Hsp27 but also a variety of other proteins. Therefore, the conflicting evidence seen in these two reports may be due to the fact that Hsp27 localises to mitochondria during heat shock, a translocation that may be dependent on the synthesis of other proteins induced by activation of HSF-1.

Recently however, Paul et al. (Paul et al., 2002) have reported that while Hsp27 localises to the mitochondrion, this localisation of Hsp27 alone is not sufficient to prevent cytochrome c release and that Hsp27-mediated prevention of cytochrome c release was due to the ability of Hsp27 to maintain integrity of the actin network and prevent the translocation of pro-apoptotic factors from the actin cytoskeleton to the mitochondrion where they can trigger the release of cytochrome c (Paul et al., 2002).

A thorough investigation of the significance of the mitochondrial pool of Hsp27 remains to be carried out. The idea that the protective roles of Hsps are exerted at the level of the mitochondrion is not an entirely new concept. It was previously suggested by Polla and co-workers that on exposure to oxidative stress, mitochondria are the targets of the protective effects of Hsps (Polla et al., 1996). In addition, it has also been shown in PC12 cells, that a novel set of sHsps are localised to the mitochondria where their role is to protect cells against thermal and oxidative stresses (Downs et al., 1999). These findings add further support to the suggestion that the mitochondrion may be the primary site for the protective effects of Hsp27. As the mitochondrion plays such a key role in the execution of apoptosis it would seem a suitable location for Hsp27 and other Hsps to elicit their anti-apoptotic characteristics.

In addition, Hsp27 has also been shown to interact with another component of the apoptotic cell death machinery, pro-caspase-3 (Concannon et al., 2001; Pandey et al., 2000).

This interaction would appear to be with the inactive form of the molecule and therefore it is highly plausible to suppose that the binding of Hsp27 to pro-caspase-3 prevents its activation, possibly by preventing initiator caspases, such as caspase-9, from acquiring access to the necessary residues whose cleavage results in activity of the enzyme. The elucidation of critical residues, on both of these molecules, which are responsible for their interaction should aid in further characterizing the role this interaction may play, if any at all, in regulation of apoptosis. Interestingly, an interaction with pro-caspase-3 has also been shown to occur for α-B crystallin, another of the sHsps (Kamradt et al., 2001).

It appears as though Hsp27 is adapted to inhibit apoptosis induced by a variety of different mechanisms as Hsp27 expression is also associated with inhibition of apoptosis initiated by the binding of death ligands to cell surface receptors such as Fas (Mehlen et al., 1996). The activation of Fas signalling is not predominantly associated with cytochrome c release and apoptosome formation. In this case receptor binding leads to the direct activation of pro-casapse-8 with the subsequent downstream activation of pro-caspase-3. As it has been shown that Hsp27 interacts with pro-caspase-3 it may be that the function of this interaction is principally to inhibit apoptosis associated with the activation of death receptors. It has been shown, however, that Hsp27 can interact with Daxx (Charette et al., 2000), a protein implicated as being mediator of Fas-induced apoptosis (Yang et al., 1997). Apoptosis mediated by Daxx is caspase-independent and involves the recruitment of the apoptosis signal regulating kinase (Ask1), which is a MAP kinase kinase that activates JNK. Interaction of Daxx with both Fas receptor and Ask1 is blocked by interaction with Hsp27 (Charette et al., 2000). On the other hand, in some cell systems the activation of death receptors is additionally associated with amplification of the caspase cascade by way of caspase-8-mediated release of cytochrome c from the mitochondria. It could be argued that inhibition of Fas activated caspases by Hsp27 is a consequence of its ability to prevent the cytochrome c -mediated amplification of the caspase cascade. However, it seems a lot more plausible that this is mediated through a synergistic effect of interacting both with cytochrome c as well as pro-caspase-3.

### 1.8.5.5 Chaperone activity of Hsp27 and its anti-apoptotic function

For Hsp27, it appears that the chaperone activity can be dissociated from its antiapoptotic function. Hsp27 is an ATP-independent chaperone that prohibits the aggregation and promotes the refolding of denatured proteins *in vitro* as mentioned previously. *In vitro* studies have shown that the 33 amino acids of N-terminal region adjacent to the highly conserved α-crystallin domain of the protein are not necessary for its chaperone activity (Gio et al., 2000). However, it has been shown that this region is essential for cytochrome c binding and for the antiapoptotic properties of the protein (Bruey et al., 2000). Therefore, Hsps may exert the apoptosis-regulatory function, at least to some extent, through protein-protein interactions not directly related to their chaperone function. Future studies will unravel the fine mechanisms of such interactions and will provide invaluable information on the potential therapeutic role of Hsps, particularly in the context of neurodegeneration.

### 1.8.5.6 The role of Hsp27 in prevention of stress-induced disruption of the cytoskeleton

Hsp27 has been accepted as being a potent regulator of cytoskeletal dynamics particularly actin microfilaments. Within cells, the principal function of the cytoskeleton is to maintain the shape of the cell and this function is modulated both by the spatial arrangement in addition to the polymerisation dynamics of its different elements (Liang et al., 1997).

A number of studies have shown that overexpression of Hsp27 increases the stability of F-actin microfilaments on exposure to stresses such as hypothermia (Lavoie et al., 1993), oxidants (Huot et al., 1995) and cytochalasin D (Guay et al., 1997). During the course of stress, the integrity of filamentous actin structures is disturbed by the disorderly severing and aggregation of the filaments, resulting in a situation that is potentially damaging for cell morphology. During the course of heat stress, it is suggested that the association of Hsp27 with F-actin may function as an adaptive response to changes within the cellular environment, to stabilise the structure of the cytoskeleton as well as prevent its disaggregation. The exact mechanism by which F-actin is stabilised by Hsp27 is not well

understood. Turkey and murine Hsp25, which are homologues of human Hsp27, have been shown to inhibit actin polymerisation (Benndorf et al., 1994; Miron et al., 1991; Wieske et al., 2001), an activity dependent on the degree of phosphorylation of Hsp25 and on its structural organization. Non-phosphorylated monomers of Hsp25 and Hsp27 are active in inhibiting actin polymerization compared to phosphorylated monomers and non-phosphorylated oligomeric forms which are inactive (Lavoie et al., 1993; Benndorf et al., 1994). In contrast, Preville et al. (1998) have found that phosphorylation of Hsp25 is not required for protection of cells against disruption of the actin cytoskeleton and that the protection of the actin network is probably as a result of the redox change mediated by Hsp25 as opposed to a direct effect on actin by this protein. Irrespective of the mechanism by which Hsp27 inhibits actin polymerisation it would appear that the loss of Hsp27's actin-capping ability allows actin polymerisation, and as a result potentially stabilises and remodels the actin cytoskeleton in the course of stress.

Other components of the cytoskeleton, in addition to actin microfilaments, have been reported to interact with Hsp27. An interaction of Hsp27 and α-B crystallin, another member of the sHsp family, with various intermediate filaments has been reported (Perng et al., 1999). It is suggested that these interactions may manage the connections in cellular networks between filaments, an event that may be important for cell survival. There is also evidence suggesting that Hsp27 co-localizes with tubulin/microtubules, however, the significance of this interaction is not fully understood (Hino et al., 2000). It is questionable whether the association of Hsp27 with elements of the cytoskeleton promotes increased cellular survival by insulating this dynamic network and preventing its destruction, which could ultimately lead to cell death.

### 1.8.5.7 Modulation of intracellular redox potential by Hsp27

A major role in cell death is played by the generation of high levels of intracellular reactive oxygen species (ROS), which are induced by a variety of different stimuli. Electrons escaping from the electron transport chain and reacting with oxygen molecules normally occurs within cells resulting in low levels of ROS. However, on exposure of cells to certain toxic stimuli, such as TNF-α or hydrogen peroxide, there is a rapid increase in the

levels of intracellular ROS due to mitochondrial dysfunction. The ROS generated can lead to oxidative damage to the cell, potentially resulting in demise of the cell either by apoptosis or necrosis depending on the levels of ROS generated. A number of studies have demonstrated the protective effects of sHsps, such as Hsp27 and α-B-crystallin, against ROS generated through TNF-α stimulation as well as oxidative stress induced by hydrogen peroxide and menadione (Mehlen et al., 1995). These findings resulted in the theory that sHsps could function as inhibitors of ROS action, by modulating and preserving the intracellular redox potential. While Hsp27 lacks any endogenous ROS detoxifying activity, it has the ability to increase intracellular levels of glutathione (Mehlen et al., 1996), which is a tripeptide with numerous intracellular functions, including ROS detoxification and regulation of cell death. Interestingly, cells treated with agents that lower levels of glutathione, exhibit an enhancement in the induction of stress proteins including Hsp27 (Ito et al., 1998), which suggests that the expression of sHsps functions as a buffering system to prevent the oxidation of proteins, which normally occurs when there is an intracellular increase in the levels of ROS. This ability of sHsps to increase and maintain glutathione in a reduced form correlates with an increase in the activity of an enzyme involved in the ROS-glutathione pathway, known as glucose-6-phosphate dehydrogenase (Preville et al., 1999).

In summary, there is no doubt that at times of stress, the expression of Hsp27 serves as a protective mechanism to increase cellular survival. The precise mechanism of how this protection is mediated by Hsp27 is highly complex, as can be seen from the variety of different roles proposed for the protein. It is clear from these observations that the function of Hsp27 is a highly adapted and dynamic one, in which post-translational modifications play a key role. In response to a stress event, the expression of Hsp27 is only transiently induced, following which levels of expression fall drastically, thereby permitting overexpression only when its cytoprotective properties are required. Based on the evidence from a number of studies, two distinct roles have been suggested for Hsp27 during stress: (1) to sustain the normal function of cells through interaction with and stabilization of the cytoskeleton as well as by facilitating the repair or removal of damaged proteins and (Concannon et al., 2003) (2) to prevent apoptosis by obstructing caspase activation by way of an ability to sequester cytochrome c and pro-caspase-3 in addition to acting as a redox modulator (Concannon et al., 2003).

No doubt, full elucidation of the role of Hsp27 will have several potential uses as therapies for a number of various ailments. The therapeutic benefit of using exogenous Hsp27 has already been proven by a number of gene therapy experiments. Previously, Wagstaff and co-workers (1999) demonstrated that the delivery of exogenous Hsp27 to cultured neuronal cells by means of HSV-based vectors provides protection against apoptosis induced by a variety of stimuli. In addition, Kalwy et al. (2003) demonstrated the protective effects of overexpression of Hsp27, via a HSV vector, in an in vivo model of neuronal cell death. As a number of neurodegenerative diseases such as Parkinson's disease, come about as a result of excessive death of neuronal cells, some of which is apoptotic in nature, the use of gene therapy to deliver exogenous Hsp27 may help in providing a better prognosis for these diseases (Zourlidou et al., 2004). Similar effects have been shown in cardiac cells exposed to hypoxia and thermal stress (Brar et al., 1999). In addition, a number of different approaches are being developed to upregulate the endogenous levels of Hsp27 and other Hsps including the use of cytokines such as IL-2 and the use of pharmacological agents that could be administered to patients to induce enhanced Hsp expression in a non-stressful manner (Latchman, 1998).

### 1.8.5.8 Protective properties of Hsp27 in neuronal systems *in vitro*

Wagstaff et al. (1999) demonstrated for the first time that *in vitro* over-expression of Hsp27 protects primary sensory neurons and neuronal cell lines against both thermal and ischemic stress and against apoptosis induced by nerve growth factor withdrawal or serum withdrawal plus retinoic acid treatment.

More recently, *in vitro* study conducted by Shimura et al. (2004) found that Hsp27 rescued cells from pathological hyperphosphorylated tau-mediated cell death. Hsp27 preferentially bound only the pathological hyperphosphorylated tau and facilitated its degradation without ubiquitination, which otherwise does not undergo efficient degradation by the proteasome.

Wyttenbach et al. (2002) described an additional novel property of Hsp27, reporting that Hsp27 *in vitro* can suppress polyQ-mediated cell death in a cellular model of

Huntington's disease (HD). Mutant huntingtin brought about increased levels of ROS in neuronal and non-neuronal cells, resulting in cell death. Hsp27 significantly reduced ROS content in these cells, thereby providing evidence that Hsp27 may protect cells against oxidative stress. This protection conferred by Hsp27 was regulated by its phosphorylation status and was found to be independent of its ability to bind to cytochrome c. In contrast to Hsp70, Hsp27 suppressed polyQ death but not polyQ aggregation.

### 1.8.5.9 Protective properties of Hsp27 in neuronal systems *in vivo*

Another example of the neuroprotective role of Hsp27 is reflected in the study conducted in sensory neurons by Lewis et al. (1999), which showed that Hsp27 plays a role in promoting neuronal survival after axotomy *in vivo* and following NGF withdrawal *in vitro*. Their findings suggest that Hsp27 contributes significantly to the survival of sensory neurons under these conditions and in addition is likely to be an important factor for survival of adult sensory neurons too. A subsequent study by Benn et al. (2002), extended the above findings, and showed in adult rats that both motor and sensory neurons up-regulate Hsp27 in its active phosphorylated form following injury to a peripheral nerve. However, in neonatal rats the expression of Hsp27 is much lower following such injury, and only a minority of cells expressing the protein survive.

Further roles of Hsp27 in *in vivo* neuronal systems, relevant to the present study are those described by Kalwy et al. (2003) and Akbar et al. (2003). Kalwy et al. (2003) exogenously expressed Hsp27 by means of HSV-based viral vectors in the rat hippocampus and demonstrated a significant increase in survival following kainic acid administration. This was the first report demonstrating protection through exogenous expression of Hsp27 in an in vivo model of neuronal cell death. Subsequently, Akbar et al. (2003) developed transgenic mice expressing human Hsp27 at high levels in the brain, spinal cord and other tissues and tested whether Hsp27 conferred *in vivo* neuroprotection against kainite induced neuronal cell death; Hsp27 expression in the mouse brought about a reduction in kainate-induced toxicity and mortality by at least 50% and reduced neuronal cell death in the CA3 region of hippocampus, in addition to attenuating caspase-3 induction.

A recent *in vivo* study conducted by Efthymiou et al. (2004), on isolated perfused hearts from mice over-expressing Hsp27 found they were more resistant to ischemia/reperfusion injury (using infarct size as an end point) compared to hearts from non-transgenic littermates. This is the first study that demonstrates that over-expression of Hsp27 provides protection against lethal ischemia/reperfusion injury in the intact heart.

Finally, Wang et al. (2004) more recently demonstrated a further important role of Hsp27 in vivo through generation of transgenic Drosophila lines using the upstream activating sequence/GAL4 system. Over-expression of either Hsp26 or Hsp27 extended the mean lifespan of the flies by 30% and was accompanied by increased stress resistance in these flies.

### 1.8.5.10 Novel role of Hsp27 in proteasome-mediated protein degradation

Recently, a novel important role has been discovered for Hsp27 in protein degradation of certain substrates via the proteasome. Like BAG-1, HSP27 possesses an ubiquitin-like domain, enhances the catalytic activity of the 26S proteasome machinery, as well as increases the degradation of ubiquitinylated proteins in response to stressful stimuli. In contrast to Hsp70 and Hsp90, Hsp27 has been found to directly interact with ubiquitin (Parcellier et al., 2003). This ability of Hsp27 to directly interact with ubiquitin could explain the recently described co-localization of Hsp27 with ubiquitinylated proteins and the 20S proteasome in cytoplasmic inclusions that characterize a variety of degenerative diseases (Zatloukal et al., 2002).

The ubiquitin/proteasome system itself plays a part in the modulation of apoptosis. Regulatory molecules implicated in apoptosis, such as caspases through interaction with some IAPs, have been identified as substrates of the proteasome. Hsps could indirectly influence apoptosis by assisting the degradation of death regulatory proteins. NF-κB is one of these regulatory proteins whose several steps of activation involve the ubiquitin/proteasome system including maturation of the transcription factor subunits, activation of IκKs (I-κB kinases), as well as degradation of I-κB. The kinase RIP (receptor-interacting protein), which connects TNF-R1 engagement by TNFα to activation of NF-κB,

is associated with Hsp90 and geldanamycin-induced disruption of Hsp90 facilitates the proteasome-mediated degradation of RIP, thereby inhibiting NF-κB activation.

Consequently, a picture emerges in which Hsps and the ubiquitin/proteasome system work together to modulate death pathways. This cooperation may involve co-chaperones, for instance CHIP or BAG-1, whose expression may be altered in human diseases. It is expected that an improved understanding of the "protein triage" machinery will offer new avenues in the therapeutic manipulation of death pathways such as those regulated by NF-κB or IAPs.

In conclusion, recent evidence suggests a number of connections between Hsps and the apoptotic machinery. These connections involve the chaperone functions of these proteins, their ability to interact with death regulatory proteins as well as perhaps their ability to connect the protein folding machinery to the protein degradation pathway by means of interactions with co-chaperones such as those described above, CHIP and BAG-1. Further studies will reveal whether these proteins are useful targets for potential therapeutic manipulation of the apoptotic pathways.

### 1.8.6 Chaperones in neurodegenerative diseases

The accumulation of misfolded proteins in aged organisms is particularly pronounced in post-mitotic cells, such as neurons. The threat of damaged proteins increases even further, if the protein is protease-resistant The difficulties of protein degradation together with an impaired protease activity and chaperone action in aging neurons, results in a massive accumulation of these proteins and triggers neurodegeneration (Macario and Conway de Macario, 2001).

Oxidative damage and inflammatory processes are more prevalent during aging, and not only accompany but aggravate neurodegeneration (Gibson et al., 2000; Goodman and Mattson, 1994; Hemmer et al., 2001). A number of molecular chaperones are involved in the maintenance of cellular redox status (Arrigo, 1998) and work to protect neurones against oxidative stress (Lee et al., 1999; Yu et al., 1999). The involvement of Hsps in the process

of neurodegeneration and their therapeutic importance have only recently become evident. This section provides a brief account on the aspects of heat shock protein roles in a range of neurodegenerative disorders, as shown in many studies utilising cellular *in vitro* models or *in vivo* models of the diseases and human post mortem material.

### 1.8.6.1 Polyglutamine diseases

Polyglutamine repeats generate proteins, which are more susceptible to aggregation. Huntington's disease (HD) like Parkinson's disease is usually a late-onset, progressive neurodegenerative disease associated with selective neuronal loss as well as abnormal protein accumulations. HD belongs to the group of polyglutamine (polyQ) repeat diseases which also includes dentatorubropallidoluysian atrophy (DRPLA), spinal and bulbar muscular atrophy (SBMA) and spinocerebellar ataxias type 1, 2, 3 (also known as Machado–Joseph disease, MJD), 6, 7, and 17. PolyQ diseases are characterized by the expansion of glutamine within the ORF of the relevant protein. The expanded polyQ domain is toxic and consequently leads to neuronal dysfunction and degeneration.

Abnormal protein accumulation such as nuclear inclusions incorporating the disease protein and other components, have led to multiple *in vitro* and *in vivo* studies investigating the protective potential of molecular chaperones against abnormal aggregate formation and toxicity, as discussed below. Chaperones co-localize with the aggregates of these polyglutamine-containing proteins and increased levels of chaperones such as that of Hsp40, Hsp60, Hsp70, Hsc70, Hsp100 work toward inhibiting polyglutamine-containing protein aggregation and to slow disease progression (Carmichael et al., 2000; Cummings et al., 1998; Hughes and Olson, 2001; Krobitsch and Lindquist, 2000).

Several reports have demonstrated that increased expression of the Hsp70/Hsp40 chaperone system can suppress polyQ-induced neurotoxicity in fly models (Kazemi-Esfarjani and Benzer, 2000; Warrick et al., 1999; Chan et al., 2000; Fernandez-Funez et al., 2000) and also in a mouse model of polyQ disease (Cummings et al., 2001).

#### 1.8.6.1.1 Huntington's disease

Huntington's disease (HD) is an autosomal dominantly inherited progressive neurodegenerative disease. The mutant gene has been localised to chromosome 4p16.3 with the gene product huntingtin found to be widely distributed in both neurones and extraneuronal tissues. Huntington's disease comes about as a result of a mutation resulting in the expansion of a trinucleotide (CAG) repeat encoding glutamine. As yet the etiology of Huntington's disease remains unknown but increasing evidence suggests important role of altered gene transcription, mitochondrial dysfunction and excitotoxicity. The expanded polyglutamine stretch results in a conformational change and abnormal protein-protein interactions. Mutant huntingtin can bind to transcription factors, resulting in reduced levels of acetylated histones. One of the consequences of this appears to be a decreased expression of genes, which may play critical roles in neuronal survival.

*In vitro* experiments carried out in yeast by Muchowski et al. (2000) demonstrated that Hsp70 and Hsp40 can interact with mutant huntingtin and inhibit the formation of detergent insoluble fibrillar aggregates. Sittler et al. (2001) first demonstrated with geldanamycin that huntingtin protein aggregation in cells can be suppressed by chemical compounds activating a specific heat shock response. Geldanamycin is a benzoquinone ansamycin that binds and inhibits heat shock protein Hsp90 and triggers a heat shock response in mammalian cells; at nanomolar concentrations geldanamycin induces the expression of Hsp40, Hsp70 and Hsp90 and inhibits HD exon 1 protein aggregation in a dose-dependent manner in mammalian cells.

Some other examples include the study by Dedeoglu et al. (2002) where striatal lesion sizes were evaluated in homozygous and heterozygous Hsp70 over-expressing mice and wt controls receiving 3-nitropropionic acid or malonate. Mice over-expressing Hsp70 exhibited increased resistance to malonate and 3-nitropropionic acid. Malonate and 3-nitropropionic acid are well-characterized inducers of animal models of HD, which inhibit succinate dehydrogenase, leading to mitochondrial dysfunction and triggering the generation of superoxide radicals, secondary excitotoxicity and apoptosis.

However, studies conducted by Hansson et al. (2003) in a mouse model of HD, found that Hsp70 has only modest effects on disease progression. Hansson et al. (2003) crossed R6/2 mice, expressing exon 1 of the HD gene with an expanded CAG repeat, with mice over-expressing Hsp70. The resulting R6/2-Hsp70 transgenic exhibited a 5- to 15-fold increase in Hsp70 expression in neocortical, hippocampal and basal ganglia regions; this correlated with a delayed loss of body weight compared to R6/2 mice. However, overexpression of Hsp70 did not affect the number or size of nuclear inclusions, the loss of brain weight, reduction of striatal volume, reduction in size of striatal projection neurons, development of paw clasping phenotype or early death of the mice. In addition it was noted that in older R6/2-hsp70 mice a large proportion of the Hsp70 protein was accumulated in nuclear inclusions.

Recently, Hay et al. (2004) reported a progressive decrease in Hdj1, Hdj2, Hsp70, alphaSGT and betaSGT brain levels in the R6/2 mouse model of HD. All these proteins were found to co-localise with nuclear but not with extranuclear aggregates. The mRNA level of Hdj1 and alphaSGT do not change, therefore it is possible that the decrease in protein levels is a result of their sequestration into aggregates, or possibly due to an increase in protein turnover, as a result of their relocation to the nucleus. It was found that Hsp70 over-expression in the R6/2-Hsp70 transgenic delayed aggregate formation by one week but had no effect on the detergent-solubility of aggregates and did not alter the course of the neurological phenotype. However it was shown through organotypic slice cultures from transgenic HD mouse that pharmacological agents, radicicol and geldanamycin, both induced and maintained various Hsps for at least three weeks, and altered the detergent soluble properties of polyQ protein aggregates (Hay et al., 2004).

Finally, *in vitro* experiments conducted by Wyttenbach et al. (2002) reported that Hsp27 suppressed polyQ-induced ROS formation in cells caused by huntingtin and conferred protection to neuronal and non-neuronal cells against poly-Q-mediated toxicity without reducing polyQ-protein aggregation. These findings demonstrate that oxidative stress brought about by polyQ expansion contributes to cell death and suggests an important new role for Hsp27 in preventing toxicity associated with polyQ expansions.

#### 1.8.6.1.2 Hsps and other polyQ diseases

As mentioned above, other polyQ diseases include dentatorubropallidoluysian atrophy (DRPLA), spinal and bulbar muscular atrophy (SBMA) and Machado–Joseph disease (MJD). Adachi et al. (2003) cross-bred spinal and bulbar muscular atrophy (SBMA) transgenic mice with mice over-expressing human Hsp70 and demonstrated amelioration of the disease phenotype and found that the nuclear localisation of the mutant androgen receptor (AR) as well as of monomeric mutant AR was reduced, suggesting a possible Hsp70-mediated AR degradation. In a previous study, in an *in vitro* cellular system, it had been shown that overexpression of Hsp70 and Hsp40 resulted in an enhanced mutant AR solubility and proteosomal degradation as well as a decrease in the mutant AR half-life (Bailey et al., 2002).

In spinocerebellar ataxia type 1 (SCA1), which is characterized by loss of motor coordination as a result of degeneration of cerebella Purkinje cells and brain stem neurons, the expanded protein aggregates into nuclear inclusions containing chaperones, ubiquitin, and proteosomal subunits (components of the protein refolding and degradation machinery). Cummings et al. (2001) crossbred SCA1 mice with inducible Hsp70 over-expressing mice to determine whether enhancing chaperone activity may alleviate the phenotype in a mouse model by reducing protein aggregation. Although the amount of nuclear inclusions in Purkinje cells remained unchanged, further analysis revealed that high levels of Hsp70 protect against neurodegeneration and preserved dendritic arborisation in the cerebellum.

Interestingly, it has been found that Hsp104 and its bacterial homolog, ClpB, can solubilise small protein aggregates in collaboration with Hsp70/Hsp40modulators of polyQ aggregation. Direct interaction of the prokaryotic ClpB (Hsp104) and DnaK (Hsp70) in the ATP-dependent resolubilization of aggregated proteins has been reported with the chaperone complex described thought to facilitate the transfer of intermediates between ClpB and DnaK during refolding of substrates from aggregates (Schlee et al., 2004).

### 1.8.6.2 Chaperones and Alzheimer's disease

Alzheimer's disease is the best-known example of folding-related neurodegenerative disease. Alzheimer's disease (AD) is a neurodegenerative disorder characterised by the relentless decline of cognitive function, alterations in judgement, perception and personality, and ultimately the loss of essential qualities that define a human existence. The many signs and symptoms of AD are the sign of the dysfunction of diverse brain regions. The histopathological hallmarks of AD are numerous senile plaques and neurofibrillary tangles in specific regions of the brain. Senile plaques comprise an extracellular core of aggregated, fibrillar β-amyloid peptide (Aβ), which is accompanied to varying degrees by microglial cells, astrocytes, and dystrophic neuronal processes (Hauw et al., 2001; Cummings et al., 2004). Aβ is cleaved from the β-amyloid precursor protein (βAPP) by enzymes generically named β-secretase (or β-amyloid cleaving enzyme, BACE) and γ-secretase (Cummings et al., 2004; Hardy et al., 2002). Neurofibrillary tangles (NFT) consist of intracellular fibrils, which are composed of aberrantly polymerised, hyperphosphorylated *tau*, a protein that normally participates as a monomer in the assembly and stabilisation of microtubules (Lee et al., 2001). In addition to plaques and tangles, an elaborate and inconsistent mixture of other lesions are found in the AD brain, such as cerebral β-amyloid angiopathy, granulovacuolar degeneration, neuropil threads, Lewy bodies, and selective but widespread degeneration of neurons and their connections (Hauw et al., 2001; Cummings et al., 2004).

A number of studies demonstrated the induction of small heat-shock proteins (Hsp27, crystallin), Hsp70 and ubiquitin (a 6kDa heat-shock protein, which labels damaged proteins and directs them for proteolytic degradation) in neurons affected by Alzheimer's disease and in surrounding astrocytes. Neuronal chaperones have been found to be localized in neuritic plaques and neurofibrillary tangles (Cisse et al., 1993; Hamos et al., 1991; Perez et al., 1991; Renkawek et al., 1993; Shinohara et al., 1993).

In the affected neuron in Alzheimer's disease, the accumulated chaperones participate in an attempt to sequester the β-amyloid and other damaged proteins (Hamos et al., 1991; Kouchi et al., 1999). However, αB-crystallin the small heat-shock protein enhanced the neurotoxicity of the amyloid-β 1-40 peptide probably by maintaining it in a

nonfibrillar, highly toxic form (Stege et al., 1999). Cytoplasmic Hsp60, which is a specific chaperone for actin and tubulin, is found to be decreased in Alzheimer's disease-affected neurons resulting in both the deficiency and aggregation of cytoskeletal proteins (Schuller et al., 2001). Non-affected nerve cells of Alzheimer sufferers, such as olfactory neurons (Getchell et al., 1995) also exhibited a reduction in expression of Hsp70.

In Alzheimer's disease (AD), which is a progressive amnesic dementia there is evidence of post-translational hyperphosphorylation, enzymatic cleavage, and conformational alterations of the microtubule-associated protein tau. A recent study by Petrucelli et al. (2004) showed that CHIP and Hsp70 regulate tau ubiquitination, degradation and aggregation. CHIP was found to interact directly with the microtubule-binding domain of tau, induce tau ubiquitination, increase the levels of insoluble aggregated tau and interestingly in addition, CHIP was found to be present in tau lesions in human post mortem brain tissue. The same study found that the levels of tau were reduced in mice over-expressing Hsp70 and that Hsp70 induction through geldanamycin or HSF-1 led to a decrease in steady-state tau levels and in detergent insoluble and hyperphosphorylated tau. Previously, Shimura et al. (2004) reported tau binding to Hsc70, and also the requirement for phosphorylation prior to ubiquitination by CHIP. CHIP rescues phosphorylated tau-induced toxicity; however the study by Petrucelli et al. (2004) further explores the opposing action of Hsp70 and CHIP on tau ubiquitination and aggregation. CHIP, Hsp70, parkin, and Pael-R (see section 1.8.6.3 below on PD) have been shown to form a complex both *in vitro* and *in vivo* with the amount of CHIP in the complex increasing during ER stress (Imai et al., 2002). CHIP was shown to promote the dissociation of Hsp70 from parkin and Pael-R, thereby facilitating parkin-mediated Pael-R ubiquitination. Finally, Dou et al. (2003) found that in transgenic mice and in AD brains tau aggregation is inversely related to levels of Hsp70 and Hsp90 and that these elevated levels are responsible for enhanced tau solubility and it's binding to microtubules.

### 1.8.6.3 Chaperones and Parkinson's disease

Parkinson's disease is an age-related disorder characterized by a progressive degeneration of dopaminergic neurons in the substantia nigra and demonstrating a

corresponding motor deficit. An increasing amount of evidence shows that in addition to oxidative stress and mitochondrial dysfunction, defects in protein folding are also key elements of Parkinson's disease etiology. Similar to Alzheimer's disease, glial and astroglial cells of Parkinson's disease sufferers exhibited the expression of αB-crystallin and as with the neurofibrillary tangles, aggregated proteins in Lewy bodies had a large content of various heat-shock proteins (Jellinger, 2000). Dietary restriction induced expression of Hsp70 and Grp78 and paralleled a protective effect in a Parkinson's disease model (Duan and Mattson, 1999). Interestingly, the protein parkin, whose mutations trigger the autosomal recessive juvenile parkinsonism was identified as an ubiquitin-ligase playing an important role in the degradation of ER misfolded proteins, such as a G-protein coupled membrane receptor, called Pael and synphilin, an α-synuclein interacting protein (Chung et al., 2001; Imai et al., 2001).

As reviewed by Bonini (2002), molecular chaperones were the first modifiers described to hinder the progression of neurodegeneration in Drosophila. Hsp70 was identified as a potent suppressor of disease phenotype in both HD and PD in flies. Specifically, Hsp70 expression in Drosophila prevented dopaminergic neuronal loss associated with α-Syn. In addition, Auluck et al. (2002) demonstrated that interference with the endogenous chaperone activity in flies accelerated α-Syn toxicity. Interestingly, although increased expression of Hsp70 suppressed α-Syn toxicity, it did not alter the microscopic appearance of neuronal inclusions.

Auluck et al. (2002) also reported the presence of Hsp70 and Hsp40 in LBs and LNs of post mortem PD brain. LBs, which are the neuropathological hallmarks of the disease, were shown to contain many other Hsps such as Hsp27, αB-crystallin, torsinA, Hsp90, Hsp110 and ubiquitin. The presence of Hsps in LBs provides an additional link between Hsps and the disease, however as yet, their role in the process of neurodegeneration remains unclear.

Recently, Klucken et al. (2004) crossed α-Syn and Hsp70 transgenic mice and found that Hsp70 reduced high molecular weight and detergent insoluble α-Syn species but did not

change the total amount of soluble monomeric α-Syn. This same study reported Hsp70 over-expression provided protection against α-Syn toxicity in an *in vitro* cellular model of α-Syn aggregation (human H4 neuroglioma cells) in addition to a reduction of detergent insoluble α-Syn species. It was suggested by Auluck et al. (2002) that Hsp70 protection from α-Syn toxicity can be dissociated from prevention of α-Syn aggregation. It would therefore be interesting to investigate whether overexpression of Hsp70 in the double transgenic mouse, besides reducing aggregation, could in addition ameliorate dopaminergic cell death and improve the disease phenotype of the model described by Klucken et al. (2004).

### 1.8.6.4 Chaperones in Amyotrophic Lateral Sclerosis

In addition to the various previous reports on the role of Hsps in neurodegeneration there are a few important recent studies related to Hsps in various ALS models and in post mortem ALS brains. These studies have been described above in the previous sections of the Introduction of this thesis (Bruening et al., 1999; Vleminckx et al., 2002; Batulan et al., 2003; Kieran et al., 2004). These studies highlight the potential of Hsps as therapeutic targets for this disease, but further work is necessary for a better understanding of the role of Hsps in ALS pathology. Evidence from the abovementioned ALS studies and other neurodegenerative disease models, suggests that pharmacological agents that induce the expression of molecular chaperones in neurons could be a fruitful strategy for the treatment of ALS.

In a transgenic mouse model of this disease, over-expressing human mutant SOD-1, arimoclomol drug treatment resulted in a reduction in the rate of disease progression, associated with Hsp induction (Kieran et al., 2004). Arimoclomol is a hydroxylamine derivative that acts as a co-inducer of Hsp expression and was found to successfully increase HSF-1 activation in the spinal cord of arimoclomol treated mice and resulted in a corresponding increase in the protein levels of Hsp70, Hsp90 and to a lesser extent, Hsp27. Importantly, arimoclomol treated mice exhibited significant improvement in motorneurone survival and hind limb muscle function and exhibited a 22% increase in lifespan.

In other studies, in cultured neurons, mutant SOD-1 toxicity was delayed by gene transfer of Hsp70 (Bruening et al., 1999). Interestingly, αB-crystallin and Hsp27 were found to be upregulated in the spinal cord of mutant SOD-1 mice but not mice over-expressing wt SOD-1, while Hsp70 levels were normal (Vleminckx et al., 2002). Finally, Batulan et al. (2003) demonstrated the existence of a high threshold for induction of the stress response in motor neurons due to their reduced ability to activate HSF-1.

Neurodegenerative diseases such as ALS, PD, AD and the prion diseases all have the assembly of structurally unrelated proteins into intracellular or extracellular amyloid fibrils in common. Therefore, since all the intermediates that are formed early in these diseases can be highly cytotoxic for the neurons (Bucciantini et al., 2002; Walsh et al., 2002), it is possible that there might be common mechanisms in amyloid formation and toxicity, and hence common strategies for the development of neuroprotection.

These above studies highlight the therapeutic potential of Hsps and raise the possibility that Hsps may be used as therapeutic agents in neurodegenerative diseases. However further work is necessary to fully understand the role of Hsps in ALS pathology. Evidence from the above mentioned studies, in the context of neurodegenerative diseases and ALS, suggest that agents possessing the pharmacological ability to induce the expression of molecular chaperones in neurons could provide an effective strategy for the treatment of ALS and other neurodegenerative diseases.

In view of the multiple functions of Hsps, in addition to suppressing protein misfolding and aggregation, it is evident that manipulation of the Hsp expression in animal models is a potential starting point to develop neuroprotective strategies for ALS and other similar neurodegenerative diseases. In summary, ALS is characterised not only by aggregation of protein but also mitochondrial dysfunction, formation of reactive oxygen species, oxidative stress and cell death. Hsps have been implicated in all the above pathways, as mentioned earlier in the introduction of this thesis, as a consequence Hsps comprise eligible candidates for neuroprotection in ALS.

Therefore it was decided to investigate the effects of Hsps against mutant SOD1 associated toxicity. This work looks at the mechanism by which mutant SOD1 triggers cell death and the effects of Hsp27 and/or Hsp70, singly and in combination, against the damaging effects of mutant SOD1 in both a neuronally-derived ND7 cell line and primary cell culture systems.

## 1.9 Aims and Objectives

The aims of the project, for which results are presented in this thesis, were as follows:

- To characterize and utilize an *in vitro* mutant-SOD1 toxicity cellular model system in order to test various hypotheses with regards to the effect of wt and FALS-associated-SOD1 mutants in neuronal cells.

- To test the protective role of Hsps, in particular Hsp27 and Hsp70, against mutant-SOD1 associated toxicity under various stresses paralleling conditions relevant to FALS in the above *in vitro* system.

- To subsequently investigate the underlying mechanisms of the protective effects of Hsps in this model.

The following three Chapters describe:

- The characterization of this model system and discuss its limitations and advantages in relation to the literature as well as suggest further experiments (Chapter 3).

- The utilization of this model system and HSV-based vectors to study the neuroprotective properties of Hsps (Chapter 4). Along with preliminary investigations into the mechanism of the protection provided by Hsp27 and/or Hsp70 and the effects of ApoE on this model system (Chapter 4)

- The creation of a primary cell culture (DRG) model system by means of HSV-based vectors incorporating wt-SOD1 and G93R-mutant-SOD1 to further study the neuroprotective properties of Hsps and investigate their mechanism of protection (Chapter 5).

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Laboratory Reagents

### 2.1.1 General Suppliers

General analytical grade laboratory chemicals were purchased from the following companies: Sigma Chemical Company Ltd., Poole Dorset, UK; BDH Merck Ltd., Lutterworth, Leicestershire, UK; Boehringer Mannheim, Lewes, East Sussex, UK. Phosphate buffered saline (PBS) was made up from PBS tablets (1 tablet in 500ml ddH2O) purchased from Invitrogen Ltd, Paisley, UK. All solutions were made up using MilliQ 18MΩ water (Millipore), which was autoclaved where necessary. General laboratory plasticware was purchased from BDH Merck Ltd. and Ependorff, Cambridge, UK.

### 2.1.2 Cells, Viruses and Transgenic mice

The SOD1 stable cell lines and SOD1 viruses were constructed in our laboratory by Dr Yolanda Collaco-Moraes. The Hsp viruses were constructed in our laboratory by Dr Marcus Wagstaff. The transgenic Hsp27 (Tg-Hsp27) mice were a gift from Professor J. de Belleroche, Imperial College, London, UK. The Hsp70 transgenic mice (Tg-Hsp70) were a gift from Dr. C.E. Angelidis, Dalhousie University, Canada. The Hsp27 transgenic mice incorporate a transgene containing human Hsp27 cDNA with a chicken - actin promoter and cytomegalovirus enhancer (pCAGGS) (see Akbar et al., 2003). The Hsp70 transgenic mice incorporate human inducible Hsp70 gene under the regulation of β-actin promoter (see Plumier et al., 1995). The ApoE protein isoforms were a gift from Dr B.S. Thilakawardhana, Royal Free Hospital, University College London.

### 2.1.3 Molecular Reagents and Plasmids

All the restriction endonucleases and DNA modifying enzymes and their respective buffers were supplied by Promega Southampton, UK; except for the enzyme BamH1 which was purchased from New England Biolabs, UK. For PCR, nucleotides, buffers and Taq Polymerase were obtained from Bioline, London, UK. The DNA 1kb molecular weight marker was purchased from Invitrogen Ltd, Paisley, UK. The altered site II *in vitro*

mutagenesis system was purchased from Promega, Southampton, UK. DNA sequencing was performed by MWG-Biotech AG (Ebserberg, Germany). The plasmid vectors used were: pcDNA3neo from Invitrogen Ltd, UK, into which SOD1 cDNA was cloned. Human fibroblast SOD1 cDNA was obtained from ATCC (Accession no: #61646).

### 2.1.4 SDS-PAGE Reagents

Acrylamide/bisacrylamide (30% w/v) solution for polyacrylamide gels was purchased from Amresco Ltd., Ohio, USA. Protein molecular weight Rainbow™ marker, Hybond™-C nitrocellulose membranes, Enhanced Chemiluminescence system (ECL) and Kodak X-OMAT imaging photographic film were all purchased from Amersham Pharmacia Biotech, Little Chalfont, Bucks., UK. The photographic chemicals for developing and fixing were purchased from X-OGRAPH Ltd., Tetbury, UK.

### 2.1.5 Antibodies

Hsp27 antibody, (goat polyclonal; product no: SC-1049; diluted 1:1000 in 4% milk/PBS) and β-actin antibodies (goat polyclonal; product no: SC-1616; diluted 1:1000) were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Hsp70 antibody (mouse monoclonal; product no: SPA-810; diluted 1:1000 in 4% milk/PBS) and the SOD1 (rabbit polyclonal antibody; product no: SOD-100; 1:1000) was from Stressgen Ltd., UK. Secondary horseradish peroxidase-linked antibodies used for Western immunoblotting were all from DAKO Ltd., Glostrup, Denmark; anti-mouse IgG biotinylated (Cat no. BA-9200) and anti-rabbit IgG biotinylated (Cat no. BA-1000).

### 2.1.6 Tissue Culture Reagents

All tissue culture media and reagents were purchased from Invitrogen Ltd, Paisley, UK unless otherwise stated. All plasticware used was obtained from Nunc Ltd., Roskilde, Denmark unless otherwise stated. Sterile 0.2μM and 0.45μM disposable filters were obtained from Millipore, Watford, UK. All trans-Retinoic acid was purchased from Sigma was resuspended in dimethylsulfoxide (DMSO) and aliquoted into stocks of 3 mg/ml and

kept in -80°C freezer until required, minimizing all exposure to light. Antibiotics Geneticin® (G418-sulphate) and Zeocin™ were purchased from Invitrogen Ltd, Paisley, UK. All caspase-inhibitors Z-VAD-FMK (inhibits caspase -1-like proteases), Z-IETD-FMK (irreversible caspase-8 inhibitor), Z-LEHD-FMK (irreversible caspase-9 inhibitor) were obtained from Calbiochem®; all were solubilised in DMSO as per supplier protocol and stock solutions of 50mM, 10mM and 20mM prepared, respectively; aliquots were kept at -20°C and used at a final concentration of 10μM. Hexamethylene bisacetamide (HMBA) was purchased from Sigma and solubilised in double distilled water, filtered sterilized and kept in -4°C until required. HMBA was used at a final concentration of 3 mM.

### 2.1.7 Cell Death Detection and Quantification

Trypan blue was purchased from Sigma Chemical Company Ltd, Poole, Dorset, UK. TUNEL (terminal dUTP nick end labelling) reagent was purchased from Roche Molecular Biochemicals, GmbH, Mannheim, Germany.

### 2.1.8 Equipment

The Trans-Blot™ cell transfer tanks and the Bio-Rad GS-800 densitometer were purchased from Bio-Rad Laboratories Ltd., Hertfordshire, UK.
The Zeiss microscope was purchased from Carl Zeiss Mico-Imaging Inc., New York, USA.
Labsystems Multiskan RC plate reader, Finland.
The Beckman Centrifuge was purchased from Beckman Coulter Inc., California, USA.
All gas cylinders were supplied by the British Oxygen Company, BOC Ltd, Guildford, UK.

## 2.2 Cell Culture

Media and reagents were all sterile on purchase or sterilised by either autoclaving or filtering through 0.2μm filters. All cell culture work was carried out under sterile conditions in a laminar flow cabinet. Cells were passaged 25 times after which they were discarded. All tissue culture preparations were carried out under sterile conditions in a Laminar flow safety cabinet. All viral preparations were carried out in line with Health and Safety Executive category 2 conditions.

### 2.2.1 Cell Biology

Cell lines were frozen down in 1ml aliquots and stored in liquid nitrogen until needed and were maintained at 37°C while in culture in either a 5% $CO_2$ incubator in a humidified atmosphere or on a rotary roller apparatus (for roller bottles). All cell manipulations were carried out under sterile conditions by means of standard aseptic techniques.

### 2.2.2 Mammalian Cell Lines and Growth Media

**ND7 Cells** A hybrid cell line created by the fusion of immortalised HGPRT-mouse neuroblastoma cells (N18Tg2) with rat post-mitotic neonatal dorsal root ganglion neurons (Wood et al., 1990). ND7 cells were cultured in Liebovitz L15 media containing 10% foetal calf serum (FCS), 100 units $ml^{-1}$ penicillin and streptomycin, supplemented with 0.35% (w/v) D(+)-glucose, 2mM L-glutamine 100x and 0.375% (w/v) sodium bicarbonate. Wild-type SOD1, G93A, G93R and empty vector control ND7 stable cell lines were created previously by Dr. Yolanda Collaco-Moraes. These stably transfected cell lines were cultured in the presence of 800μg $ml^{-1}$ of Geneticin-G418 sulphate (Invitrogen) and SOD1 expression was monitored prior to experimentation by western blotting.

**MAM49** Transformed baby hamster kidney cells (BHK) cell line, a fibroblast cell line grown in Dulbecco's modified Eagle's medium containing 10% FCS and 100 units $ml^{-1}$ penicillin and streptomycin and cultured in the presence of 800μg $ml^{-1}$ of G418 and 175μg

$ml^{-1}$ zeocin selection to maintain two plasmids, one containing the viral gene ICP4 and the other containing viral gene ICP27.

### 2.2.3 Growth Conditions and Storage of Mammalian Cell Lines

Cells were grown in $80cm^2$, $175cm^2$ flasks, or $800cm^2$ roller bottles in the appropriate growth medium (see section 2.2.2). All cell lines were passaged when they were 80-90% confluent and not used for more than 25 passages. ND7 cells were passaged by pouring off the old media and replacing with 10ml of fresh media. Cells were dislodged and the resulting cell suspension was aliquoted into fresh culture flasks to a final dilution of 1:10 in fresh growth media.

BHK MAM49 cell line was passaged by washing in Hanks Balanced Salt Solution (HBSS) at room temperature, and incubating for 2-3minutes with 5ml per $175cm^2$ culture flask of 10% (v/v) trypsin in versene at 37°C/5% $CO_2$. The effect of trypsin was neutralised by adding 5ml of growth media, the cells were vortexed to prevent clumping, and split 1:10 in fresh growth media.

For long-term storage, frozen stocks were made by pelleting the cell suspension from one $175cm^2$ flask. The cell pellet was resuspended in a 1:1 mix of 1ml of 20% FCS in appropriate cell media and 1ml of 15% dimethylsulphoxide (DMSO; Merck Ltd, Poole, Dorset, U.K.) in appropriate media and transferred to 2x 1.5ml cryotubes. The vials were then transferred to -80°C freezer for 2 weeks, in an insulated box to ensure gentle freezing and subsequently to liquid nitrogen. For recovery, frozen cells from one vial were rapidly thawed at 37°C and transferred immediately to 15ml of growth media, and pelleted in a standard bench-top centrifuge at 20°C, 1,000rpm for 5 minutes. The resulting pellet was resuspended in 10ml of appropriate fresh growth media and seeded into $T25cm^2$ TC flasks with appropriate concentration of selection for respective cell line and passaged once 80% confluent.

### 2.2.4 SOD1 and SOD1-mutant stable cell lines

The SOD1 stable cell lines were created in our laboratory by Dr Yolanda Collaco-Moraes. Human wild type SOD1 or SOD1 mutant cDNA was inserted into pCDNA3neo mammalian expression vector under control of a CMV promoter and transfected into ND7 cells using the calcium phosphate procedure. Briefly, ND7 cells were grown in 6-well plates at a density of approximately 10,000 cells per well and 5μg per well of the expression vector incorporating wt-SOD1, G93A or G93R-mutant-SOD1 as well as empty pCDNA3neo vector control were transfected using the calcium phosphate method (Stow and Wilkie, 1976; Gorman, 1986). G418-sulphate was added to the media 48h post transfection at final concentration of 800μg/ml (Smith et al., 1997; Reeves et al., 1999). After 5-10 days approximately 80-100 individual G418-resistant colonies per construct were isolated using a micropipette under the x40 objective of a light microscope under sterile conditions in a laminar flow cabinet. The media was replaced every 2-3 days with fresh G418-sulphate containing media. Stable cell lines were produced, with clones selected being neomycin resistant. Clones expressing either wt or mutant SOD1 were identified by means of Western blot.

## 2.3 Induction of Cell Stress

### 2.3.1 Serum withdrawal (plus 1μM all-trans retinoic acid)

ND7 cells undergo apoptosis when subjected to serum-withdrawal and addition of all-trans retinoic acid (Howard et al, 1993).

**Apoptosis Media:** 50% DMEM
50% Ham's F- 12 medium
5μg $ml^{-1}$ human transferrin
250ng $ml^{-1}$ bovine insulin
30nM sodium selenite
10μM all-trans retinoic acid (stock dissolved in DMSO)

The wt-SOD, ND7 empty vector control and G93A and G93R mutant stable cell lines were rinsed gently with 1ml well$^{-1}$ of 1 x PBS. They were subsequently incubated in 1ml well$^{-1}$ apoptosis media for 24 and 48 hour time points at 37°C/5% $CO_2$. The cells were harvested at the appropriate time points and cell death assessed by Trypan blue exclusion (see Section 2.6.1) and number of apoptotic cells determined by TUNEL analysis (see section 2.6.2).

For serum-withdrawal and all-trans retinoic acid addition experiment retinoic acid was added to final concentration of 1μM per well with cell death being assessed by Trypan blue exclusion as before (section 2.6.1) and the number of apoptotic cells were determined by TUNEL analysis (section 2.6.2).

### 2.3.2 IFN-γ treatment, Staurosporine and Camptothecin administration

Optimal concentrations of IFN-γ, staurosporine, camptothecin, hydrogen peroxide and glutamate were determined by death curves; the concentration used of each substance is provided below under each separate insult.

Cells were plated out so as to attain 70% confluency the following day, in 6 well plates. The cells were rinsed gently with 1ml well$^{-1}$ of 1 x PBS and 2ml of fresh ND7 growth media added per well. IFN-γ was then added to each well to a final concentration of 50ng/ml. Cells were gently harvested, centrifuged and the pellet resuspended in 1x PBS at 24h and 48h time points and cell death immediately assessed by trypan blue exclusion assay and by terminal dUTP nick end labelling (TUNEL) (see section 2.6).

For staurosporine experiments, 70% confluent ND7 cells were rinsed gently with 1ml well$^{-1}$ of 1 x PBS and 2ml of fresh ND7 growth media added per well. Staurosporine in DMSO (Sigma), was added to a final concentration of 1μM per well. Cells were gently harvested, centrifuged and the pellet resuspended in 1x PBS at 2h, 4h, and 6h and 8h time points and cell death immediately assessed by trypan blue exclusion assay and by terminal dUTP nick end labelling (TUNEL) (see section 2.6).

For camptothecin experiments, 70% confluent ND7 cells were rinsed gently with 1ml $well^{-1}$ of 1 x PBS and 2ml of fresh ND7 growth media added per well. Camptothecin in DMSO (Sigma), was added to a final concentration of 1μM per well. Cells were gently harvested, centrifuged and the pellet resuspended in 1x PBS at 24h and 48h time points and cell death immediately assessed by trypan blue exclusion assay and by terminal dUTP nick end labelling (TUNEL) (see section 2.6).

### 2.3.3 Hydrogen Peroxide and Glutamate Treatment

Cells were plated out so as to attain 70% confluency the following day, in 6 well plates. The cells were rinsed gently with 1ml $well^{-1}$ of 1 x PBS and 2ml of fresh ND7 growth media added per well. Hydrogen peroxide was then added to each well at a final concentration of 10μM. Cells were gently harvested, centrifuged and the pellet resuspended in 1x PBS at 24h and 48h time points and cell death immediately assessed by trypan blue exclusion assay and by terminal dUTP nick end labelling (TUNEL) (see section 2.6).

For glutamate experiments, 70% confluent ND7 cells were rinsed gently with 1ml $well^{-1}$ of 1 x PBS and 2ml of fresh ND7 growth media added per well. Glutamate was added to a final concentration of 5μM per well. Cells were gently harvested, centrifuged and the pellet resuspended in 1x PBS at 24h and 48h time points and cell death immediately assessed by trypan blue exclusion assay and by terminal dUTP nick end labelling (TUNEL) (see section 2.6).

### 2.3.4 Simulated Ischemia Followed by Re-oxygenation

Ischemia can be simulated *in vitro* by incubating the cells in a physiological buffer containing raised levels of lactic acid and potassium, and decreased pH along with inhibitors of electron transport and glycolysis. This method is based on one devised by Esumi et al., 1991 for simulating ischemia upon cardiomyocytes and has been further adapted by Dr. Jing Zhao (The Rayne Institute for Cardiovascular Studies, United Medical and Dental Schools, London, UK) and used successfully in our laboratory on cells lines and Dorsal root ganglia (DRG) neurons by Dr Marcus Wagstaff.

**Control Buffer:** 118mM NaCl
24mM $NaHCO_3$
4mM KCl
1mM $NaH_2PO_4$
2.5mM $CaCl_2$
1.2mM $MgCl_2$
0.5mM EDTA
2mM sodium pyruvate
10mM D-glucose
pH 7.4

**Ischemic Buffer:** Control Buffer containing:
20mM sodium lactate
12mM KCl
pH 6.2

Both mutants, wt-SOD and empty vector control ND7 stable cell lines were plated out in 6 well plates so as the wells were 70% confluent the following day. Once 70% confluent the cells were rinsed gently with 1ml $well^{-1}$ of 1 x PBS. 1ml of ischemic buffer was then added to each well and the plate placed in an ischemic chamber 37°C/5% $CO_2$ for 4 hours. The cells were then taken out the chamber and the ischemic buffer was removed and 2ml of fresh appropriate growth media added to each well.

After 24 hours the cells were harvested and cell death assessed by Trypan blue exclusion and by terminal dUTP nick end labelling (TUNEL) (see Section 2.6).

### 2.3.5 Caspase inhibitors

All caspase-inhibitors, zVAD-fmk (inhibits caspase-1-like proteases), zIETD-fmk (irreversible caspase-8 inhibitor) and zLEHD-fmk (irreversible caspase-9 inhibitor) were purchased from Cabiochem and were dissolved in DMSO. The cells were incubated for 1h with the inhibitors at a final concentration of 10μM, prior to experimentation. DMSO alone

was used in the non-caspase inhibitor treated cells at all times; as the inhibitors were dissolved in DMSO. Following incubation the cells were subjected to a range of death insults described above. After 24 hours the cells were harvested and cell death assessed by Trypan blue exclusion and by terminal dUTP nick end labelling (TUNEL) (see Section 2.6).

## 2.4 HSV-based Viral Vectors

### 2.4.1 Production of high titre stock of recombinant virus

The viruses used in this study were constructed and characterised Dr Marcus Wagstaff. Hsp or green fluorescent protein (GFP) under the control of cytomegalovirus immediate early (CMV-IE) promoter were introduced into a disabled HSV vector (Wagstaff et al., 1999). The Hsp cDNAs incorporated into the disabled HSV vector were Chinese hamster Hsp27, rabbit Hsp56, and inducible human Hsp70. The viral vectors lack the gene encoding the essential HSV immediate early protein ICP27 and hence were grown on a complementing transformed Baby Hamster Kidney (BHKs) cell line that had been stably transfected to express ICP27 (Howard et al., 1998), thereby allowing lytic growth of the virus and enabling the preparation of high titre viral stocks.

A 175cm$^2$ tissue culture flask containing the appropriate cell line grown at 37°C/5% $CO_2$ until 70-80% confluent was infected with 1x10$^6$ plaque forming units (pfu) of an initial stock of virus (from 2.2.8). Infection occurred in 10ml serum free DMEM Media for 1 hour after which viral replication was allowed by addition of 10ml growth media supplemented with 3mM HMBA. Cells were dislodged by vigorous shaking and harvested 3-5 days later and stored at -80°C as an intermediate viral stock.

For large-scale viral culture of wild type and partially disabled vectors, 10x175cm$^2$ flasks of 100% confluent cells were split into 10x850cm$^2$ roller bottles (Corning Glass Works, Corning, New York, USA) and grown in 100ml of growth media without selection at 37°C/5% $CO_2$. Once 80% confluent, the old media was poured off and cells were infected with 1x10$^5$-1x10$^6$ pfu of virus per RB (from the intermediate viral stock) and grown in 50ml

of fresh growth media supplemented with 3mM hexamethylene bisacetamide (HMBA) without selection at 32°C/0.5rpm for 3-5 days until complete CPE was observed. HMBA was added in order to induce immediate early gene transcription in the absence of virion protein VP16 (VMW65), a coat protein required for infection, for HSV mutant 1764 viral DNA (Ace et al., 1989). Cells were dislodged and harvested with supernatant by vigorous shaking of the roller bottle and immediately frozen at -80°C. After defrosting, the cellular debris was removed by centrifugation in a standard benchtop centrifuge at 3500rpm for 45minutes at 4°C. The supernatant was then removed and filtered through a 0.45μm filter (necessary to minimise debris carry-over) and then spun at 12000 rpm for 2 hours at 4°C in a Beckman JA10 rotor in pre-autoclaved pots. The supernatant was poured off and the pots inverted in the tissue culture hood for approximately 3-5minutes. The subsequent viral pellet was gently resuspended in a minimum amount of serum-free DMEM (typically 100-150μl per 250ml pot). The resuspended pellet was then sonicated for 10 seconds in a water bath sonicator and chilled on ice between each sonication for a minute. This process of sonication was repeated 5 times until the solution was completely homogeneous, after which the virus was aliquoted into cryotubes in 20μl aliquots and stored at -80°C. All Virus stock was titred as in section (2.4.3) and over-expression of the appropriate gene to be delivered to the cells confirmed.

### 2.4.2 Viral Infection of Cells

Cells were infected with recombinant viruses for subsequent *in vitro* assays. Virus was added to 70% confluent cells in 0.5ml $well^{-1}$ (6-well plate) or 0.25ml $well^{-1}$ (24-well plate) of serum free DMEM and plates were incubated at 37°C/5% $CO_2$ for 1 hour. The viral media was taken off after the hour and replaced with 2ml $well^{-1}$ (6-well plate) or 1ml $well^{-1}$ (24-well plate) of appropriate full growth media. Cells were incubated overnight at 37°C/5% $CO_2$, prior to each assay. The virus efficiency was approximately 95-100%. The HSV viral vector used in the case of SOD1 viruses also expressed GFP enabling successfully infected cells to be monitored under UV light. All virus work was carried out in a categorised room in class 11 safety cabinets and all resulting waste such as pipette tips, media and plastic-ware was disinfected in 1% virkon for a minimum of 2 hours prior to autoclaving followed by incineration.

Infection titre of virus on the stable cell lines was defined by multiplicity of infection (m.o.i. = number of pfu per cell; m.o.i. optimised by titration). ND7 cell lines were infected at an m.o.i. of 10 pfu $cell^{-1}$.

### 2.4.3 Titration of Virus on Complementing Cells

MAM49 cells complementing HSV-1 ICP4 and ICP27 were seeded in a 24 well plate and grown at 37°C/5% $CO_2$ to 80% confluency. A 1: 10 serial dilution was made of the virus from $1x10^{-2}$ - $1x10^{-10}$ml and added to the wells each containing 0.5ml of serum free DMEM. The plate was incubated for 1 hour at 37°C/5% $CO_2$. After 1hour the media was taken off by pipette and replaced with 2ml 1:2 of 1.6% (w/v) carboxymethyl cellulose (CMC): growth media supplemented with 3mM HMBA. The cells were then returned to the incubator for a further 48 hours at 37°C/5% $CO_2$. The numbers of plaques in each well were counted under the microscope in order to determine virus titre in plaque forming units per ml (pfu/ml). Hsp expressing viruses had no reporter gene for visualisation of the virus so titration was performed as described for the other viruses containing reporter genes except that infected cells were observed under light microscopy and "white" plaques were counted in the cell monolayer on the culture dish. A viral plaque is as a patch of cells, which display a distinct morphology and can be easily distinguished; within a viral plaque the infected cells are rounded up and attached to the plate with most still alive. Viruses expressing green fluorescent protein (GFP) as a reporter gene were detected by simply viewing the infected cells under a microscope with UV light, with GFP positive cells fluorescing green.

### 2.4.4 Assessment of efficiency of gene delivery

Efficiency of gene delivery in cells infected with viruses expressing green fluorescent protein GFP reporter gene was calculated under an inverted fluorescent microscope by means of UV light at a wavelength of 520nm. The infected green cells expressing GFP were counted in relation to the total number of cells present in the well and the percentage efficiency of gene delivery was then calculated.

### 2.4.5 Purification of Viral Recombinants by Plaque Selection

After UV light visualisation as in Section 2.4.4 green plaques were picked from the monolayer using a P20 Gilson micropipette set at 5µl underneath an inverted fluorescent microscope. The green fluorescent cells were isolated using a pipette tip and sucked up into the pipette and transferred to a freezing vial containing 100µl of serum free DMEM. The resulting suspensions were then freeze-thawed three times. 10µl and 90µl of the mix were then added to separate 35mm-diameter wells of 80% confluent MAM49 cells each containing 0.5ml of serum free DMEM. The cells were incubated for 1 hour at 37°C/5% $CO_2$ prior to addition of CMC/FGM and incubation for 48 hours as in Section 2.6.5. In order to purify the viruses by cloning this 2-day cycle of picking, infecting and visualising was carried out until all infected plaques appeared green under UV light. The whole well was harvested by scraping and after three cycles of freeze-thawing, a sample (100µl) of the mixture was added to non-complementing BHKs as above and examined by microscopy for any viral plaques indicating replication. The 'master' stock with no detectable activity was then titrated and infected onto a 24-well plate as in Section 2.4.3 and plaques counted and the pfu (number of plaque forming units per ml of viral suspension) calculated.

### 2.4.6 Treatment of Cells after Viral Infection

Cells were infected with virus 24h prior to stress (for virus infection: see section 2.4.2; for induction of cell stress: see section 2.3). Efficiency of virus infection was assessed prior to stress (see section 2.4.4). Following subjection of cells to stress, cells were gently harvested, centrifuged and the pellet resuspended in 1x PBS at 24h and 48h time points and cell death immediately assessed by trypan blue exclusion assay and by terminal dUTP nick end labelling (TUNEL) (see section 2.6).

## 2.5 Analysis of Protein Levels

### 2.5.1 SDS-polyacrylamide gel electrophoresis and Immunoblotting

**SDS-PAGE**

12% acrylamide gels were used to resolve proteins of molecular weight less than 50 kDa. The gel composition and buffers were adapted from Sambrook et al., (1989).

| | |
|---|---|
| **6 x SDS gel loading buffer:** | 300mM TrisHCl (pH6.8) |
| | 600mM DTT |
| | 10% SDS |
| | 30% glycerol |
| | 0.1% bromophenol blue |
| **5 x Tris-glycine electrophoresis buffer:** | 25mM Tris.HCl |
| | 250mM Glycine |
| | 0.1% SDS |

Composition of 12% polyacrylamide resolving gels and stacking gel (in ml; total volume 10 ml):

| | **Resolving 12%** | **Stacking gel** |
|---|---|---|
| $H_2O$ | 3.3 | 6.8 |
| 30% Acrylamide | 4.0 | 1.7 |
| 1.5 M Tris.HCl (pH 8.8) | 2.5 | - |
| 1.0 M Tris.HCl (pH 6.8) | - | 1.25 |
| 10% SDS | 0.1 | 0.1 |
| 10% Ammonium persulfate | 0.1 | 0.1 |
| TEMED | 0.004 | 0.01 |

For immunoblotting of cell lysates, between 20-50µg of total protein was loaded on the gels in a total loading volume of between 10-20µl. Equal loading of protein samples was

confirmed by performing a Bicinchoninic (BCA) Protein Assay (see section 2.5.4). The appropriate volume of 6xSDS loading buffer was added to each sample and the total loading volume adjusted by addition of cell lysis buffer. The samples were denatured in a heating block by heating at 95°C for 5min prior to loading. The gel plates were cleaned with $dH_2O$ and 70% ethanol prior to use. The appropriate percentage acrylamide resolving gel was prepared and poured into the gel assembly and over-layered with water saturated isopropanol to create a level interface. After polymerisation of the resolving gel had taken place the isopropanol was poured off and the top of the gel rinsed a few times with $dH_2O$ to remove the isopropanol. The stacking gel was then poured on top of the polymerised resolving gel. The protein samples along with high range molecular weight protein standards (Rainbow Markers, Amersham International Plc, Little Chalfont, Bucks, UK) were loaded on to the gel. The large polyacrylamide gels were electrophoresed in the appropriate gel electrophoresis apparatus with Tris-Glycine electrophoresis running buffer at 180Volts, 40mA per gel, for 5-6 hours or until the dye front had reached the base of the gel.

### 2.5.2 Transfer of protein

**Western transfer buffer (pH 8.3):** 25 mM Tris.HCl
192 mM Glycine
20% (v/v) Methanol

The protein samples, which had been run and separated on the gel, were then transferred to nitrocellulose filters. A nitrocellulose membrane and 6 pieces of 3MM (Whatman) paper were cut to the same size as the SDS-page gel. A stack was formed inside the minicell cassette consisting of three pieces of 3MM paper, the SDS-page gel, the nitrocellulose filter and the remaining three pieces of 3MM paper, all of which were presoaked in transfer buffer; the side containing the nitrocellulose membrane was placed nearer the anode. Transfer was carried out in a Bio-Rad Trans-blot$^{TM}$ Cell protein transfer apparatus at 200mA for 6h at room temperature or at 100mA overnight at 4°C.

### 2.5.3 Immunodetection

Membranes were blocked in blocking buffer (1x PBS, 4% (w/v) skimmed milk powder, 0.1% polyoxyethylene sorbitan monolaurate - Tween 20) for 1 hour at room temperature by shaking gently on a shaker platform. The blocking buffer was removed and the membrane was then incubated with primary antibody diluted in 4% (w/v) skimmed milk powder in 1x PBS for a period of 1 hour at room temperature on the shaker (see section 2.1.4 for details of primary antibodies used). The membrane was washed for 10 minutes in wash buffer (1x PBS, 0.1% Tween 20) by constant shaking on the shaker at room temperature and this was repeated three times to wash off any unbound antibody. The membrane was then incubated at room temperature on the shaker with the appropriate anti-IgG horseradish peroxidase (HRP) conjugated secondary antibody diluted to the required concentration in 4% block buffer (see section 2.1.4 for details of secondary antibodies used). Any unbound secondary antibody was removed by washing the membrane three times for 10 minutes in wash buffer at room temperature on a shaker. The bound horseradish peroxidase was then detected using enhanced chemiluminescence (ECL™, Amersham International Plc, Little Chalfont, Bucks, UK) according to the manufacturer's protocol and the resulting light emissions were visualized by exposure to X-ray film for 5 seconds-1 hour, depending on the strength of the signal. Membranes were stripped by submerging the membrane in stripping buffer and incubating at 50°C for one hour with occasional agitation, prior to re-blocking and re-probing.

**Western blot antibody stripping buffer**

100mM β-mercaptoethanol
2% SDS
62.5mM Tris.HCl (pH 6.7)

### 2.5.4 Protein Assay

The amount of protein in the samples was quantified by means of a Bicinchoninic (BCA) Protein Assay Reagent Kit, according to the manufacturer's protocol. In brief, bovine serum albumin (BSA) standards were prepared and cell lysates diluted using 10µl lysate and 40µl

loading buffer in the absence of bromophenol blue or DTT. 50µl standards and cell lysates were added to each well of a 96 well microplate along with 200µl of BCA working reagent and the plate incubated for 1h at 37°C. The absorbance was measured at 560nm on a plate reader (Labsystems Multiskan RC plate reader) using Labsystems Genesis Communication software, and the protein concentration was calculated from the standard curve.

## 2.6 Cell Viability Assays

### 2.6.1 Trypan Blue Exclusion Assay

Cell survival was assessed by the trypan blue exclusion assay. Trypan blue assay relies on the ability of living cells to exclude the dye and hence remain opaque whereas non-viable cells are unable to exclude the dye and therefore stain blue. Each of the stable cell lines were harvested and spun at 1000rpm for 5 mins. The remaining cell pellet was then resuspended in 200µl of 1 x PBS (phosphate-buffered saline). 20µl of each sample were mixed gently with the same volume of 0.04% (w/v) Trypan Blue (Sigma Chemical Company Ltd, Poole, Dorset, U.K.). The suspension was incubated at room temperature for about 2-3 minutes and 10µl of this mix were then loaded on a haemocytometer counting chamber (Weber Scientific International Ltd., U.K.) and the number of viable/non viable cells counted on a haemocytometer counting chamber at 40x magnification. Similar numbers of total cells (live/dead) were observed in each case. 200-300 cells were counted and classified as live or dead in each case.

### 2.6.2 TUNEL

Programmed cell death, or apoptosis, was detected using the fluorescent in situ cell death detection kit, supplied by (Roche Molecular Biochemicals, GmbH, Mannheim Germany). Labelling of 3'-hydroxyl ends of DNA fragments was carried out using terminal deoxynucleotidyl transferase (TdT) and rhodamine-conjugated nucleotides (Roche Molecular Biochemicals, GmbH, Mannheim Germany). The kit enables detection of DNA fragmentation by labelling breaks in DNA strands using terminal deoxynucleotidyl

transferase (TdT) to incorporate nucleotide polymers containing fluorescein-conjugated dUTP. This TdT-mediated dUTP nick end labelling (TUNEL) assay can therefore be used to allow detection of individual apoptotic cells in cultures of cells in chamber slides. The method used was modified slightly from that supplied with the kit.

ND7 stable cell lines and DRGs were fixed in 4% paraformaldehyde (dissolved in PBS by heating at 60°C and adjusting the pH to 7.4 by using 10M NaOH) for 10 minutes and 15 minutes at room temperature. The cells were then washed carefully three times with PBS and then permeabilized with 0.1% Triton X-100 in PBS for 10 minutes at room temperature. The cells were then washed twice with PBS and 100µl of TUNEL reaction mix was added (containing calf thymus TdT, reaction buffer and labelled nucleotide polymers in accordance with manufacturer's protocol) to the ND7 stable cells and 50µl to the DRGs and the plates incubated in the dark at 37°C for 60 minutes and 90 minutes respectively. After 60 and 90 minutes respectively the TUNEL reaction mix was removed and the reaction stopped by adding PBS with 1mM EDTA, for 5min at room temperature. The ND7 stable cells were then washed twice with PBS and stored in PBS in the dark at 4°C until microscopy. The coverslips with DRGs were washed twice with PBS mounted onto slides and stored in the dark overnight at 4°C until microscopy.

Volumes for TUNEL labelling for one well of 24-well plate:

| | |
|---|---|
| 5x Reaction Buffer | 20µl |
| 25mM $CoCl_2$ | 10µl |
| 25mM labelled dUTP | 1µl |
| 20U/µl Td Transferase | 1µl |
| Double distilled $H_2O$ | 68µl |
| | 100µl |

The samples were visualised at 40x magnification using fluorescein optics and the numbers of positive staining, apoptotic cells, in three confluent fields of view were counted for each sample. Triplicate wells/coverslips were counted per independent experiment. The number

of positive nuclei were counted and expressed as a percentage of the total cell number. Similar numbers of total cells (live/dead) were observed in each case. A minimum of 200-300 cells were counted and classified as live or dead in each case.

## 2.7 Primary Cell experiments

### 2.7.1 Genomic DNA extraction from mouse tails

All animal care and procedures were carried out in accordance with the UK Animals (Scientific Procedures) 1986 Act. Approximately 0.5cm of tail was removed from each animal and incubated in proteinase K buffer (100mM Tris, pH 8.5, 5mM EDTA, 200mM NaCl) including Proteinase K enzyme at a final concentration of 100 μg/ml. Tails were left to digest overnight, shaking at 55°C, after which half of the digest was subjected to phenol/chloroform extraction prior to ethanol precipitation. An equal volume of Tris-equilibrated phenol was added to the digest and mixed by inversion of the tube and then centrifuged at 13000 rpm in a bench top centrifuge for 10 mins at room temperature. The aqueous phase was then removed and re-extracted with an equal volume of phenol, mixed and re-centrifuged. The remaining aqueous phase was removed and subjected to chloroform extraction and re-centrifuged at 13000 rpm for 10 mins at room temperature. After this extraction the aqueous phase was added to 2 volumes of ethanol and left on ice for 30 mins and then centrifuged at 13000 rpm for 20 mins at 4°C. As much supernatant as possible was removed and the samples of DNA then allowed to air-dry overnight on the bench at room temperature. The samples were then resuspended in 40μl of sterile distilled $H_2O$ by gentle agitation for 2 hours at room temperature, so as to not shear the DNA.

### 2.7.2 Culture of primary neurons

DRG (dorsal root ganglia) sensory neuron cultures of the peripheral nervous system were prepared from newborn Harlan Sprague-Dawley rat pups at postnatal day and Hsp27 and Hsp70 transgenic mice (C57 black x ) 8-12 weeks old. The animals were sacrificed by cervical dislocation followed by decapitation.

### 2.7.3 DRG Dissection

Postnatal rats and mice were sacrificed by cervical dislocation followed by decapitation, ensuring to remove the head above the base of the spine. Using fine scissors cuts were made through the base of the spine and from the start of the neck down on each side of the animal in order to isolate the spinal cord.

Excess tissue was trimmed away from the spine, with care being taken so as not to cut into the spine. The spine was then placed in a fresh dish with the dorsal side (back) facing upwards. The spine was cut along the length to remove the top half and reveal the DRG and spinal cord. The spinal cord was discarded and No.5 forceps used to pinch out the DRGs, which were collected into 900µl HBSS in 35mm Petri dish. Dorsal root ganglia have a sheath that needs to be removed as it hinders dissociation and also introduces excess non-neuronal cells such as glia and fibroblasts into the culture. Dissociation of DRGs was as in section 2.7.5 below.

### 2.7.4 Dissecting Media

Hanks Balanced Salt Solution (HBSS), Ca/Mg free + 100units/ml penicillin and streptomycin.

### 2.7.5 Dissociation Protocol

The enzymes used for dissociating the ganglia are trypsin and collagenase (type 11), provided by Worthington (Lome Labs). Enzymes stocks were made as 10x solutions in calcium and magnesium free HBSS.

Trypsin stock was made up at 1% in HBSS, filter sterilised, and stored in 100µl frozen aliquots.
Collagenase stock was made up at 3% in HBSS, filter sterilised, and stored in 100µl frozen aliquots.

The dorsal root ganglia (DRG) cultures of both mice and rats were dissociated in a 100µl mix of 0.3% collegenase/0.1% trypsin for 25min. Following enzyme treatment 1ml of growth media was added to the dish to stop the enzyme reaction and the ganglia dissociated through either a silanised flame polished pipette or a Gilson blue tip, approximately 10 times with care being taken not to introduce air bubbles as this leads to shearing of the cells. The dissociated ganglia were then transferred to a 15ml centrifuge tube and spun at 900rpm for 5min at room temperature.

Following centrifugation supernatant was removed and the pellet carefully resuspended in appropriate growth media, see section 2.7.7. The cells were plated in 50µl droplets on laminin-coated coverslips, see section 2.7.6. Neurons were left to attach overnight in the incubator at 37°C/5% $CO_2$ and then flooded with appropriate growth media next day.

### 2.7.6 Preparing coated coverslips/dishes for culturing cells

Laminin (Sigma) is used for growth of all types of sensory, sympathetic and parasympathetic neurons. Prior to application of the laminin the coverslips were coated with Poly-D-Ornithine (pORN) (Sigma) as laminin does not adhere to uncoated plastic or glass surfaces.

A working solution of pORN was made in 0.15M Boric acid (Sigma) and sterilised by filtration through a 0.22µM filter. The coverslips were placed in a Petri dish and covered with pORN solution ensuring all coverslips were covered and then left at room temperature overnight. The next day the coverslips were washed three times with sterilised distilled water and left to stand and air-dry, in a tissue culture hood. Batches of pORN coated coverslips were made and stored in the dark at room temperature until needed.

Laminin stocks were stored in aliquots at -20°C until required. When required the laminin was allowed to thaw at 4°C in the fridge and then diluted to a final working concentration of 20µg/ml. Approximately 50µl of laminin was added to each coverslip and then left in the incubator at 37°C/5% $CO_2$ overnight. Prior to plating the cells, the coverslips were washed

twice with appropriate growth media with care being taken to prevent the laminin drying out.

### 2.7.7 Culture Media

The culture media used is a basic defined media supplemented with NFG (Nerve growth factor).

Powdered F14 (Imperial) 10x stock was made and stored at -20°C in 50ml aliquots.

Per 500ml culture media:
50ml F14 (10x stock)
450ml distilled $H_2O$
Approx. 1ml $^1/_{10}$ diluted conc. HCl (to adjust pH to 7.0)
1g sodium bicarbonate
2mM glutamine
0.35% w/v Bovine Serum Albumin (BSA)
16μg/ml putrescine
60ng/ml progesterone
38ng/ml sodium selenite
340ng/ml triiodothyronin (T3)
400ng/ml L-thyroxine

All the above constituents were obtained from Sigma apart from glutamine, which was purchased from Life Technologies.

Media was filter sterilised and aliquoted into 50ml aliquots and used for two weeks. The media was supplemented with 10ng/ml of NGF (NGF was stored in aliquots at -20°C) and 20μM cytosine β-D-arabinofuranoside (Ara-C) (Sigma), an anti-mitotic agent.

### 2.7.8 Removal of excess non-neuronal cells from postnatal preparations

Non-neuronal cells become an increasing problem with developmental age. To reduce the number of non-neuronals, the cells were spun through a column of 6% metrizamide (Sigma M3383) in culture media following enzyme dissociation. This high-density solution removes the non-neuronals, which are too small to pass through the solution. The much larger neurons pass through the solution and collect in a pellet at the bottom of the tube. After centrifugation at 900rpm for 5 minutes, the metrizamide/non neuronal mixture was tipped off and discarded.

## 2.8 Statistical Analysis

The data generated from 3-5 replicate experiments were pooled and analyzed by one-way analysis of variance (ANOVA) test, to identify differences between treatments such as over-expression of different Hsps. One-way ANOVA test is a parametric test and makes the following assumptions: the observations are independent, the sample data has a normal distribution and the scores in different groups have homogenous variances. One-way ANOVA was carried out first in order to identify any significant differences between groups. As t-tests are not recommended for multiple comparisons due to the increased risk of a type I error, as a result of more tests performed, multiple comparisons were performed with post hoc Bonferroni t-test using SPSS software. The significance level was set at $p<0.05$ unless stated otherwise. Bonferroni t-test is a modified t-test in which the P value is multiplied by the number of tests performed on the same data. This test makes the chance of obtaining significance less likely and as a result eliminates the chance of obtaining a false positive.

# CHAPTER 3

## The Effects of Wild Type and FALS-associated Mutant SOD1 Over-expression in the Neuronal ND7 Cell Line

## 3.1 Introduction

The alteration in the expression of a protein is a mechanism by which one can gain a better understanding of its biological roles. Consequently, cell lines that stably overexpress particular genes of interest are widely used to study the role of proteins in a particular cellular context. This chapter describes the establishment of an *in vitro* cellular system that allowed the study of wt-SOD1 and the disease associated mutants, G93A and G93R, which were stably over-expressed in the neuronal ND7 cell line. The advantages of the system along with some of the limitations are also discussed.

The ND7 cell line was prepared by fusion of post-mitotic rat dorsal root ganglion neurons with the N18Tg2 mouse neuroblastoma cell line, with subsequent drug selection for the resistant ganglion cells (Wood et al., 1990). This cell line is very useful as a result of its ability to proliferate indefinitely in culture whilst also retaining many of the characteristics of the neuronal cells; it can also be differentiated under certain conditions, leading to arrest of growth and outgrowth of neuronal processes. Consequently it is therefore possible to establish stable neuronal cell lines that proliferate indefinitely, over-expressing the gene of interest and enabling one to conduct multiple and reproducible experiments in large cell populations. There are also well known stresses that induce ND7 cells to undergo both apoptotic and/or non-apoptotic cell death (both of which are found to occur in the brains of ALS patients) and for this reason these cells provide a suitable model for studies of both cellular toxicity and the assessment of neuroprotection.

As discussed in Chapter 1 (Introduction), there is considerable evidence that oxidative stress and free radical mediated reactions involving superoxide, hydroxyl and peroxynitrite anions participate in the mechanism of nerve cell death in Amyotrophic Lateral Sclerosis. Important enzymes involved in the detoxification of superoxide free radicals are the mitochondrial manganese-dependent and the cytosolic and extracellular copper/zinc-dependent forms of superoxide dismutases (SOD), which convert the superoxide anion into hydrogen peroxide, which is then further detoxified by enzymes such as catalase and glutathione peroxidase. The importance of SOD in handling free radicals throughout periods of oxidative stress is well established, for example in protection against reperfusion damage in

ischemic brain (Fridovich, 1983; Imaizum et al., 1990; Liu et al., 1989; Yang et al., 1994). A role for reactive oxygen species (ROS) such as superoxide in neuronal apoptosis has also been implicated, as increased levels are detected early on following activation of apoptosis and injection of SOD1 has been shown to delay apoptosis (Estevez et al., 1998 & 2000). In addition, mutations in copper/zinc dependent SOD (SOD1) are associated with a subset of cases of familial motor neurone disease/amyotrophic lateral sclerosis (FALS) (Rosen et al., 1993). Indeed, high levels of SOD1 are found in motor neurones and nigral neurones, which may reflect the specific demands of these cell populations (Bergeron et al., 1996).

Transgenic mice over-expressing FALS causing SOD1 mutations (G93A, G93R, G37R and G85R) develop progressive paralysis with motor neurone loss (Gurney et al., 1994), thereby providing a valuable mouse model of disease although the loss of upper motor neurones typical of the human form of the disease is never observed in these transgenic mice.

The development of disease in SOD1 mutant mice is attributed to a gain of function and not reduced enzyme activity as endogenous SOD1 activity is evident and mice, which are null for SODl, do not exhibit motor neurone degeneration (Reaume et al., 1996). Several lines of study have been developed to determine the property of the mutant SOD1 bringing about the toxic gain of function which include increased tyrosine nitration, peroxidation, excitotoxicity, aggregation of SOD1, copper toxicity and reduced $Zn^{2+}$ affinity (see reviews by Cleveland and Rothstein (2001) and Beckman et al., (2001)). Mutant forms of SOD1 possess differing degrees of dismutase activity but the reduced affinity for $Zn^{2+}$ affects their catalytic activity resulting in superoxide generation from oxygen (Crow et al., 1997). The zinc deficiency (Beckman et al., 2001) could also occur in sporadic ALS cases and thus underlie this aetiology of this form of the disease where a modest zinc deficiency has already been reported (de Belleroche et al., 1987).

Evidence of apoptosis is also observed in transgenic mice overexpressing FALS associated SODl mutations from the elevated levels of caspase-1 and caspase-3 (Li et al., 2000; Pasinelli et al., 2000) and from the observation that neuroprotection can be achieved by crossing these SOD1 transgenics with mice overexpressing Bcl-2 (Kostic et al., 1997). Cell

culture experiments further substantiate the pro-apoptotic properties of FALS associated SOD1 mutations (Rabizadeh et al., 1995).

Therefore, from what was known about wt-SOD1 at the time of the establishment of the model system presented here, it was suggested that wt-SOD1 could reduce the cell death triggered by various stimuli whereas the mutants would enhance cell death under the same circumstances. The work described here adds to the literature and studies the effects of SOD1 and FALS associated SOD1 mutants in cell culture on a wide variety of death-inducing stimuli both apoptotic and nonapoptotic in order to characterise their mode of action. The two mutant forms of SOD1 chosen for this study were, G93A, which has been widely reported (Cudkowicz et al., 1997) and a second mutation, G93R, which has only been reported in a single family (Orrell et al., 1995 & 1997). These findings suggest that the disease-associated mutations appear to convert wt-SOD1 from an anti-apoptotic protein, which protects cells from cell death associated with different types of cell stress to forms, which have a universally damaging effect (Patel et al., 2002). This as well as the limitations of the system is discussed in this chapter.

## 3.2 Establishment of Cell Lines Stably Expressing SOD1 and FALS-associated SOD1 mutants

This section briefly describes the establishment and characterisation of the model system used in this study. The SOD1 stable cell lines were created in our laboratory by Dr Yolanda Collaco-Moraes. The human fibroblast SOD1 cDNA obtained from ATCC (Accession no: #61646) was cloned into the mammalian expression vector pcDNA3neo (Invitrogen Ltd, UK). This vector allowed high levels of protein expression in mammalian cells and permitted the simultaneous selection for cells with the inserted gene using G418-sulphate, as the vector also contains a neomycin resistance cassette.

Briefly, ND7 cells were grown in 6-well plates and the above mentioned expression vector incorporating wt-SOD1, G93A or G93R-mutant-SOD1 as well as empty pCDNA3neo vector control were transfected using the calcium phosphate method. G418-sulphate was added to the media 48h post transfection at final concentration of 800μg/ml

and after 5-10 days approximately 80-100 individual G418-resistant colonies per construct were isolated.

## 3.3 Identification and Characterisation of Over-expressing Clones

### 3.3.1 Western Blotting

Following colony selection, the clones that were growing normally were screened for expression of SOD1 or mutant SOD1 by means of Western blotting as described in section 2.5. Figure 3.1 shows the clear overexpression of the 16kDa SOD1 and mutant SOD1. The concentration of each of the protein samples was determined by performing a protein assay, which is described in section 2.5.4, and equal amounts of protein were loaded in each lane.

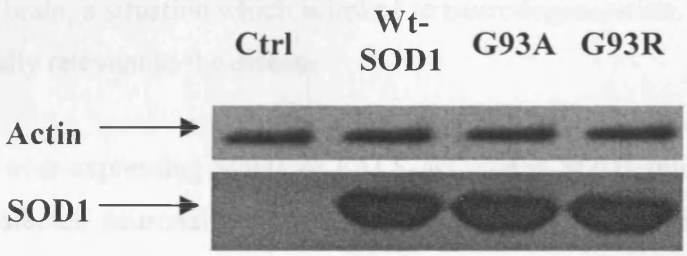



**Figure 3.1.** Level of SOD1 expression in the wt-SOD1 (lane 2), G93A (lane 3) and G93R (lane 4) mutant SOD1 cells compared to the empty vector control (lane 1) cells.

## 3.4 Responses of Cell Lines to Several Death-inducing Stimuli

The following sections describe the responses of control, wt-SOD1 and FALS-associated SOD1 mutants on exposure to various death inducing cellular stresses, such as serum withdrawal both in the presence and absence of 1μM retinoic acid, IFN-γ, staurosporine, simulated ischemia/reperfusion, glutamate, hydrogen peroxide and camptothecin (for conditions see section 2.3 of Chapter 2). Various studies have utilised stable cell lines expressing toxic or other genes of interest and through a variety of agents

investigated their responses to a range of stresses brought about by these agents, which is discussed further in the Discussion. The relevance of the stresses used here to FALS is explained in each section prior to each of the respective results obtained. Cell death was quantified by the trypan blue exclusion assay and the number of apoptotic cells assessed by TUNEL analysis as described in section 2.6.1 and 2.6.2 respectively.

### 3.4.1 Serum Removal and Serum Removal plus 1μM retinoic acid

Serum is an essential requirement for maintenance and growth of cell lines and its deletion from cell culture media is known to lead to cell death and/or differentiation (for example see Poser et al., 2003). In this present system, serum removal is used as a known apoptotic and differentiating stimulus for ND7 cells (Howard et al., 1993). It can be used to project and potentially compare the effect of deprivation of neurones from a wide range of survival signals in the brain, a situation which is linked to neurodegeneration. It is therefore used as a stress potentially relevant to the disease.

The effect of over-expressing SOD1 or FALS-associated SOD1 mutations on cell viability in stably transfected neuronal cells was investigated. Human wild type SOD1 or SOD1 mutant cDNA was inserted into the mammalian expression vector pCDNA3neo under control of a CMV promoter and transfected into ND7 cells. Expression of SOD1 was detected by means of western blotting in ND7 cells. Cells were incubated for various lengths of time in the appropriate medium in the absence of foetal calf serum and in the presence or absence of 1μM all-*trans* retinoic acid (RA) (as the retinoic acid was dissolved in DMSO it was used as a control in the absence of RA); serum withdrawal in the presence of all-trans retinoic acid induces high levels of apoptosis without significant differentiation. The response of the ND7 cell line to conditions of serum withdrawal has been well characterised in the past. ND7 cells either go through morphological differentiation into a mature neuronal phenotype with dendrite-like processes or undergo programmed cell death (Howard et al., 1993).

As seen in Figure 3.2a and 3.2b, total cell death and apoptotic cell death were measured in these cell lines following serum deprivation at 24h and 48h time points, in order to assess

the extent of cell death in the cell lines from serum withdrawal, in five independent experiments.

Total cell death was determined using the trypan blue exclusion assay in ND7 cells expressing either the vector cassette (vector control) or expressing the wild-type SOD1 gene, G93A mutant or G93R mutant gene. Cell death was found to be significantly attenuated in stable cell lines over-expressing wild-type SOD1, being decreased by 12% ($p<0.05$) and by 25% ($p<0.0005$) at 24h and 48h respectively, compared to the vector control (Figure 3.2a). On the other hand, cells expressing the G93R mutation exhibited a significantly enhanced cell death which was increased by 64% at 24h and 32% at 48h compared to a vector transfected control cell line ($p<0.0005$) and increased by 86% at 24h compared to the wild-type expressing cell line ($p<0.0005$). However, cells expressing the G93A mutation did not exhibit a large increase in death as seen with the G93R mutant, with levels of death being approximately 16% ($p<0.005$) and 8% ($p<0.0005$) at 24h and 48h respectively compared to the vector control cell line, but compared to the wild-type expressing cells at 24h and 48h, cell death was significantly increased by approximately 31% and 44% respectively ($p<0.0005$).

Apoptotic cell death following serum withdrawal was assessed using the TUNEL labelling method in ND7 cells expressing the vector cassette (vector control) or expressing the wild-type SOD1 gene, G93A mutant or G93R mutant gene (Figure 3.2b). Wild-type SOD over-expressing cells showed a significantly reduced number of TUNEL positive cells at 24h, being decreased by 16% at 24h and 27% at 48h ($p<0.0005$) while the G93R mutation brought about a 82% increase in TUNEL positive cells at 24h and 37% at 48h compared to vector control ($p<0.0005$). At the 24h time point the G93R cell line exhibited a 118% increase in TUNEL positive cells and approximately 2 times the number of TUNEL positive cells compared to the wild-type SOD1 over-expressing cells. Although the G93A mutant did not exhibit a substantial increase in cell death, cell death was approximately 22% and 8% higher at 24h and 48h respectively than vector control ($p<0.005$). Compared to wt-SOD1 cells the G93A mutant exhibited a 46% and 48% increase in cell death at 24h and 48h respectively ($p<0.0005$).

Figure 3.2a

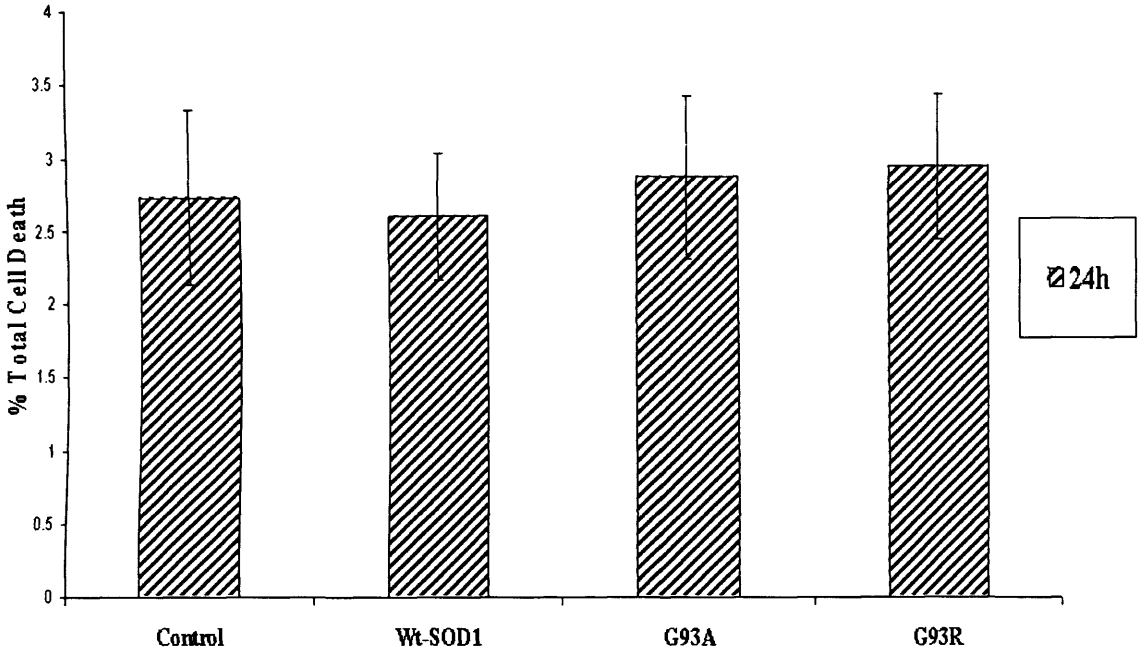



**Figure 3.2 Levels of cell death in untreated control, wild type or mutant SOD1 expressing cells at 24h.**

**a.** ND7 cell death following 24h of no treatment/no stress. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of no treatment/no stress of engineered ND7 cells expressing wt or mutant SOD1. Bars represent mean $\pm$S.D. calculated for triplicate counts per sample (n), n=5.

Figure 3.2b

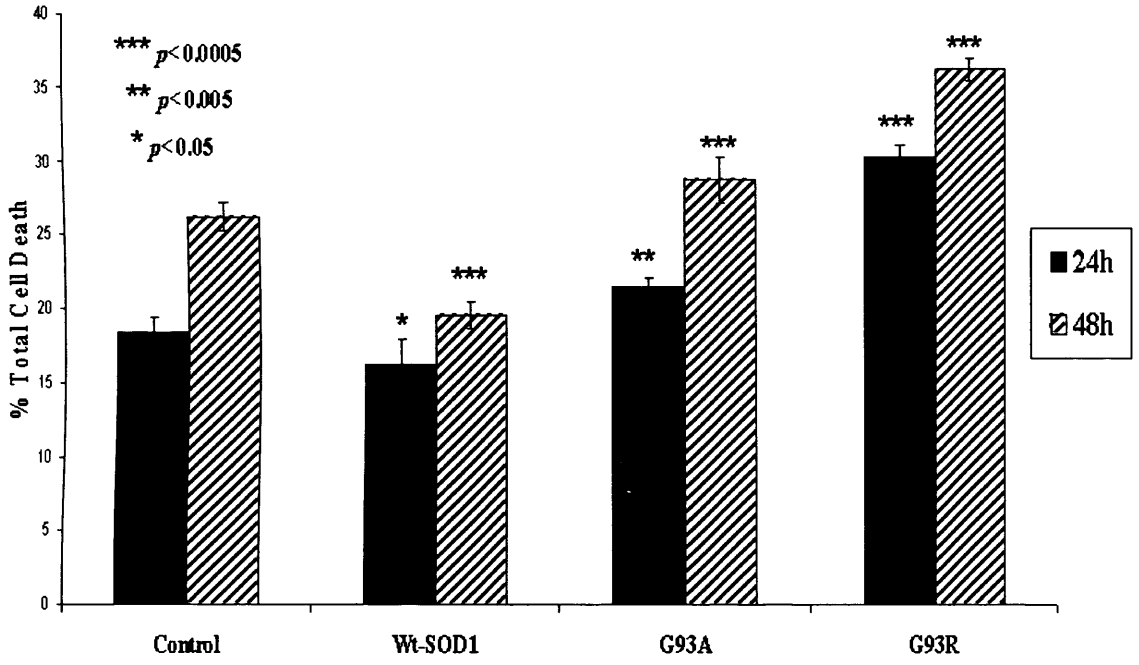



**Figure 3.2 Effect of serum withdrawal on cell death in control, wild type or mutant SOD1 expressing cells at different time points.**

**b.** ND7 cell death following 24h and 48h serum removal. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h and 48h of serum deprivation of engineered ND7 cells expressing wt or mutant SOD1. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=5. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p<0.0005$). *, **, *** indicates statistically significant difference between means, when comparing with the control ND7 empty vector cell line.

Figure 3.2c

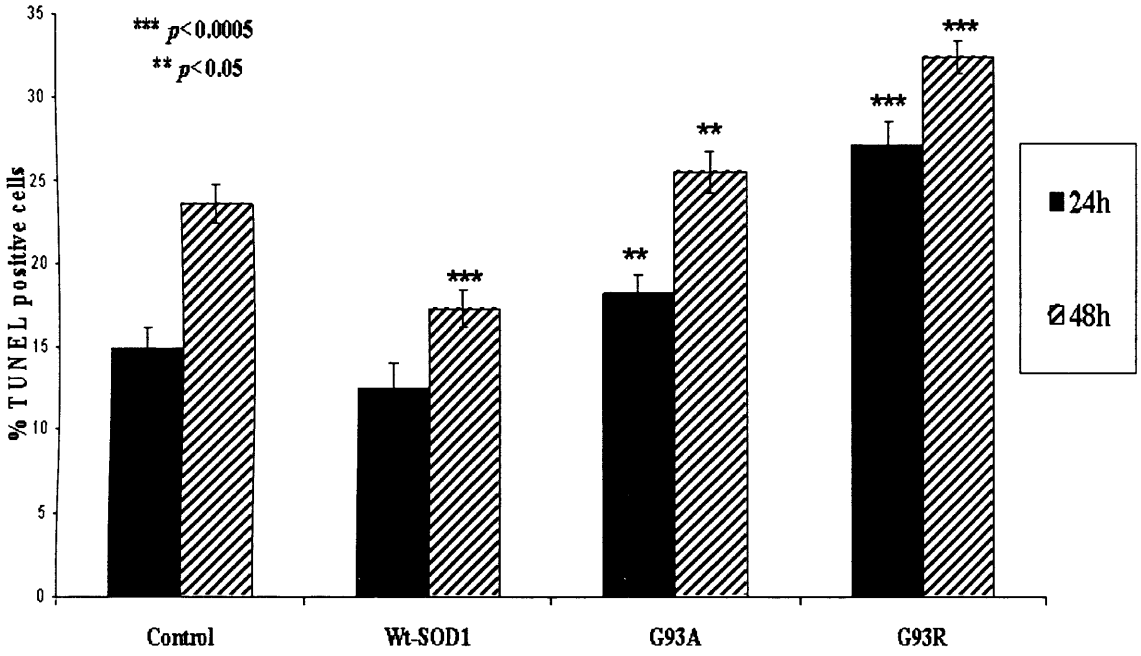



**Figure 3.2 Effect of serum withdrawal on cell death in control, wild type or mutant SOD1 expressing cells at different time points.**

**c.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were exposed to treatment of serum deprivation for 24h and 48h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=3) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with the control ND7 empty vector cell line.

On addition of 1μM retinoic acid and serum deprivation, wt-SOD1 cells exhibited a 22% and 24% reduction in death at 24h and 48h respectively compared to vector control ($p<0.0005$); (Figure 3.3a). The G93A mutant cells once again exhibited a smaller increase in cell death with death increasing by approximately 13% ($p<0.0005$) and 7% ($p<0.01$) at 24h and 48h respectively compared to vector control. The G93R mutant increased cell death by approximately 41% and 35% at 24h and 48h respectively compared to vector control ($p<0.0005$). Compared to wt-SOD1 cells the G93A cells exhibited a 44% and 42% increase in cell death at 24h and 48h respectively and the G93R cells exhibited an increase of approximately 80% and 78% at 24h and 48h respectively ($p<0.0005$).

TUNEL assessment of cell death brought about similar results. Wt-SOD1 cells exhibited a decrease of in the number of TUNEL positive cells by approximately 30% compared to vector control at both 24h and 48h time points ($p<0.0005$); (Figure 3.3b). G93A cells saw an increase in number of TUNEL positive cells by approximately 17% and 13% at 24h and 48h respectively compared to vector control ($p<0.0005$). The G93R mutant cells exhibited an increase of approximately 56% and 50% at 24h and 48h respectively compared to vector control ($p<0.0005$). Compared to wt-SOD1 cells the G93A cells exhibited an increase of 66% and 61% in the number of TUNEL positive cells and G93R cells an increase of 120% and 113% at 24h and 48h respectively ($p<0.0005$). These results from neuronal cell lines suggest that over-expressing wild type SOD1 inhibits or in some way delays apoptosis whereas the G93R mutation is proapoptotic.

In a smaller number of experiments the cell lines were incubated for the same time period in the presence of normal growth medium. A small but marked difference was observed between cell death in the various cell lines with wt-SOD1 providing a reduction in death compared to control and the mutants G93A and G93R exhibiting a slight increase in death compared to control with the G93R mutant exhibiting a slightly higher death than G93A. However, the effect of serum withdrawal on the mutants was greater even though the basal levels of death were higher.

Figure 3.3a

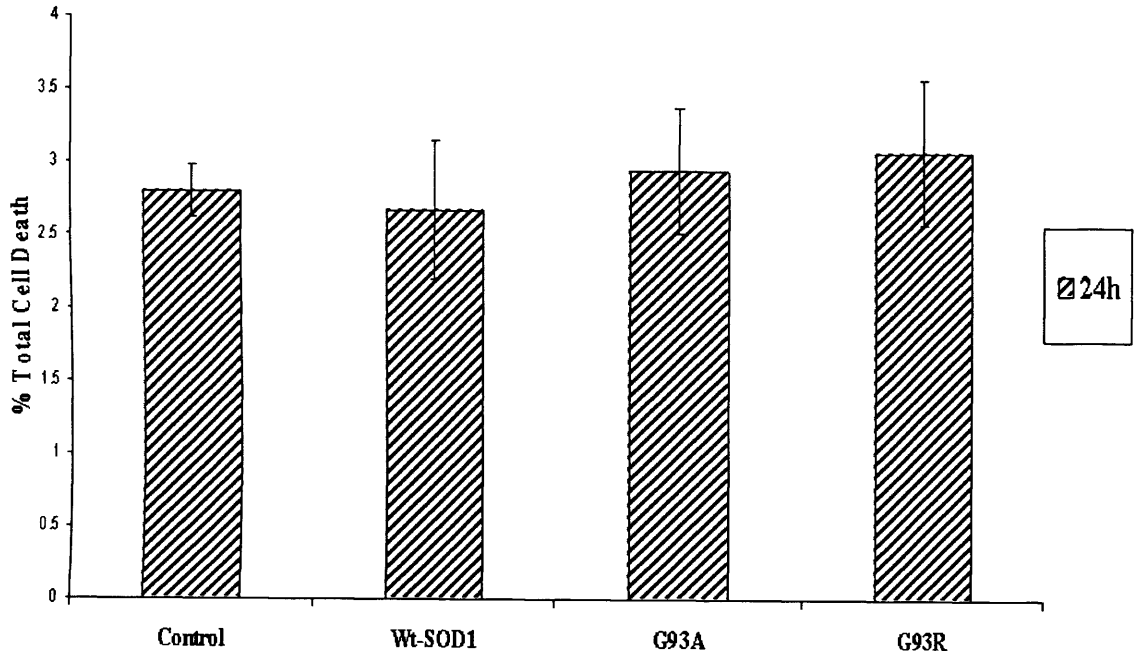



**Figure 3.3 Levels of cell death in untreated control, wild type or mutant SOD1 expressing cells at 24h.**

**a.** ND7 cell death following 24h of no treatment/no stress. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of no treatment/no stress of engineered ND7 cells expressing wt or mutant SOD1. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=5.

Figure 3.3b

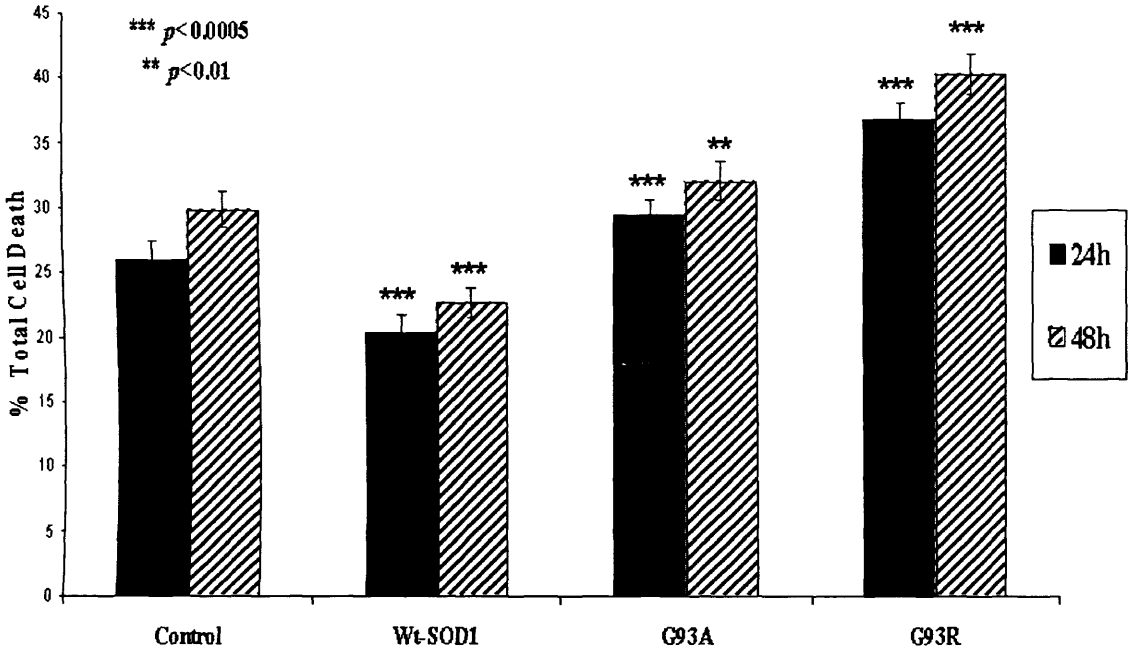



**Figure 3.3 Effect of serum withdrawal plus 1μM retinoic acid on cell death in control, wild type or mutant SOD1 expressing cells at different time points.**

**b.** Levels of total cell death in control vector, wt, mutant -G93A or G93R-SOD1 expressing cells exposed to conditions of serum deprivation for 24h or 48h in the presence of 1μM retinoic acid. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h and 48h of serum deprivation plus 1μM retinoic acid. Bars represent mean $^{+}$S.D. calculated for triplicate counts per sample (n), n=5. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p$<0.0005). *, **, *** indicates statistically significant difference between means, when comparing with the control ND7 empty vector cell line.

Figure 3.3c

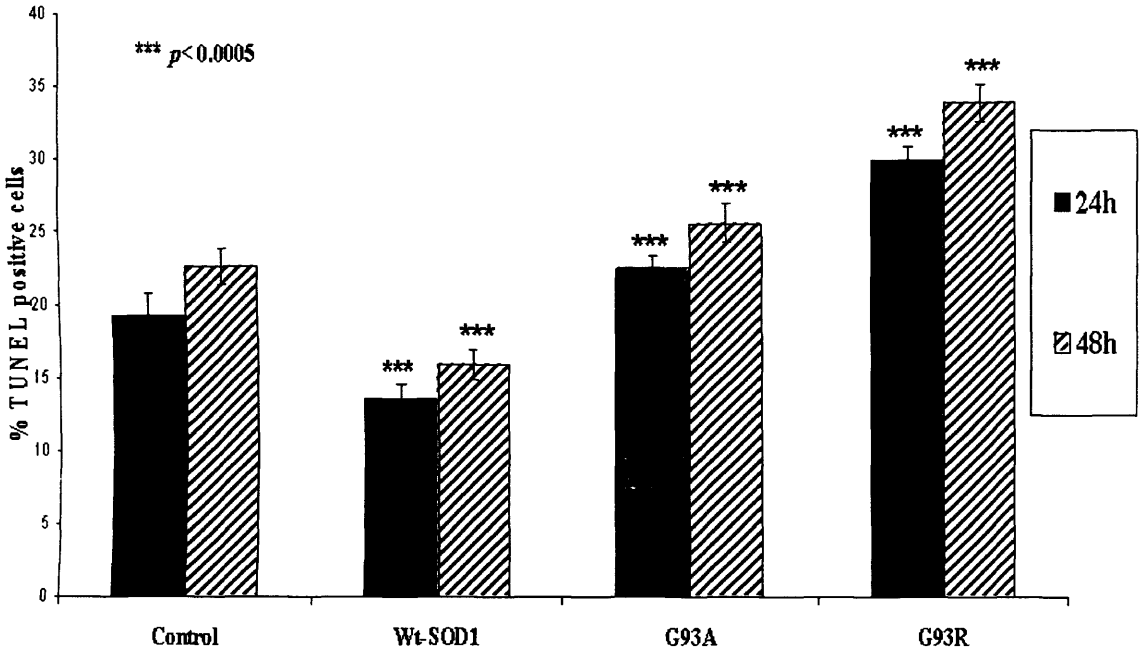



**Figure 3.3 Effect of serum withdrawal plus 1μM retinoic acid on cell death in control, wild type or mutant SOD1 expressing cells at different time points.**

**c.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were exposed to treatment of serum deprivation plus 1μM retinoic acid for 24h and 48h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=3) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with the control ND7 empty vector cell line.

### 3.4.2 Staurosporine treatment

Having established that wild type SOD1 had a protective effect against serum deprivation and serum deprivation in the presence of 1μM retinoic acid, whereas the mutants had a damaging effect, it was decided to further investigate the effect of the wild type SOD1 and the mutants on the response to other death-inducing stimuli. Therefore, a time course experiment was carried out in order to analyse the response of the different cell lines to treatment with staurosporine, which has been shown to induce apoptotic death in a variety of different neuronal and non-neuronal cell types. Staurosporine is a bacterial alkaloid that inhibits a number of cellular kinases (Gill et al., 2003) and is frequently used as an inducer of the mitochondrial apoptotic pathway. Staurosporine has been shown to induce cytochrome *c* release (Krohn et al., 1998), caspase activation (Krohn et al., 1998 & 1999), intracellular ROS accumulation (Krohn et al., 1998; Kruman et al., 1998 Prehn et al., 1997), and an increase in $[Ca^{2+}]$ (Kruman et al., 1998). Intracellular cascades activated by staurosporine depend on its concentration, with 100nM concentration inducing cell death through an apoptotic mechanism characterised by release of cytochrome *c* and a 1μM concentration of staurosporine inducing cell death through a mechanism, in which caspase activation is detected, but which shows several nonapoptotic features (Deshmukh et al., 2000). Death curves were carried out and a 1μM concentration of staurosporine was chosen to be the optimal concentration.

Total cell death was assessed by trypan blue exclusion, following 1μM staurosporine addition. Cells in 6-well plates at approximately 90% confluency were incubated in full growth media containing 1 μM staurosporine for a period of 2h, 4h and 6h. In these experiments (Figure 3.4a) it was shown that the G93R mutant clearly enhanced the degree of cell death produced by staurosporine (2-6 hours) and a similar effect of the G93A mutant was also observed, albeit less dramatic. However the wild type SOD1 showed a significant protective effect both when compared to the effect of over-expressing the mutant and also compared to the control cells containing only empty expression vector. Hence, as with the effect of serum removal and serum removal plus retinoic acid, both the mutants enhanced the damaging effects of staurosporine in comparison to the wild type protein, which

produced a protective effect. At the 2hr time point wt-SOD1 provided a protective effect with death being reduced by approximately 17% compared to control vector ($p<0.0005$). Both the G93A and G93R mutants showed an increase in cell death, at 2hr, with death increasing by approximately 18% and 32% respectively compared to vector control ($p<0.0005$). At the 4hr and 6hr time points, wt-SOD1 provided a reduction in death by approximately 12% ($p<0.005$) and 20% ($p<0.0005$) respectively, compared to vector control. In comparison the G93A and G93R mutant both exhibited increases in cell death with death increasing by approximately 30% and 21% at 4hr and 6hr respectively for G93A and 46% and 30% at 4hr and 6hr respectively for G93R mutant, compared to vector control ($p<0.0005$).

TUNEL analysis revealed similar results with the number of TUNEL positive cells being reduced in the wt-SOD1 cells by approximately 21% at 2hr compared to vector control ($p<0.05$); ((Figure 3.4b). Both G93A and G93R saw an increase in TUNEL positive cells by approximately 17% ($p<0.05$) and 48% ($p<0.0005$) respectively, compared to vector control. At 4h and 6hr time points, wt-SOD1 cells saw a reduction in TUNEL positive cells by approximately 11% and 23% ($p<0.0005$) respectively, compared to vector control. The number of TUNEL positive cells in both mutants increased at 4hr and 6hr by approximately 32% and 26% respectively for G93A and 48% and 37% respectively for G93R, compared to vector control ($p<0.0005$).

Figure 3.4a(i)

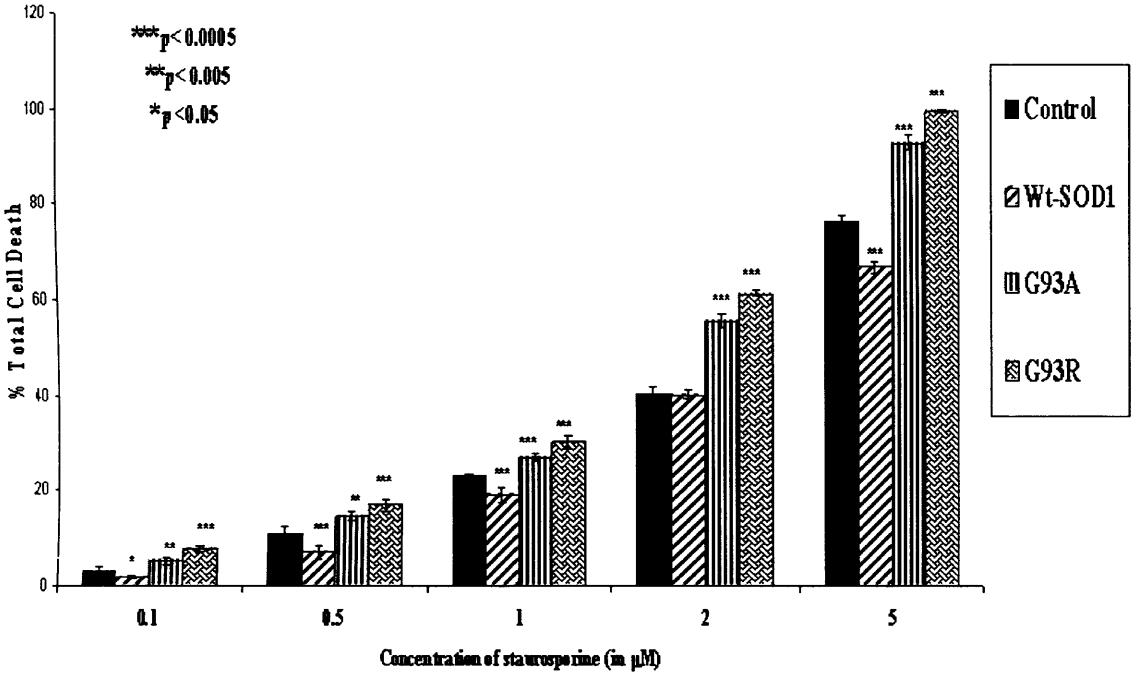



**Figure 3.4a(i) Staurosporine Kill Curve - Effect of different concentrations of staurosporine on cell death in control, wild type or mutant SOD1 expressing cells at 2h time point.**

**a(i).** Levels of total cell death in control vector, wt, mutant -G93A or G93R-SOD1 expressing cells exposed to 0.1µM; 0.5µM; 1µM; 2µM; 5µM staurosporine treatment for period of 2h. The proportion of cell death was assessed by trypan blue exclusion assay. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=5. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p$<0.0005). *, **, *** indicates statistically significant difference between means, when comparing with the control ND7 empty vector cell line.

Figure 3.4a(ii)

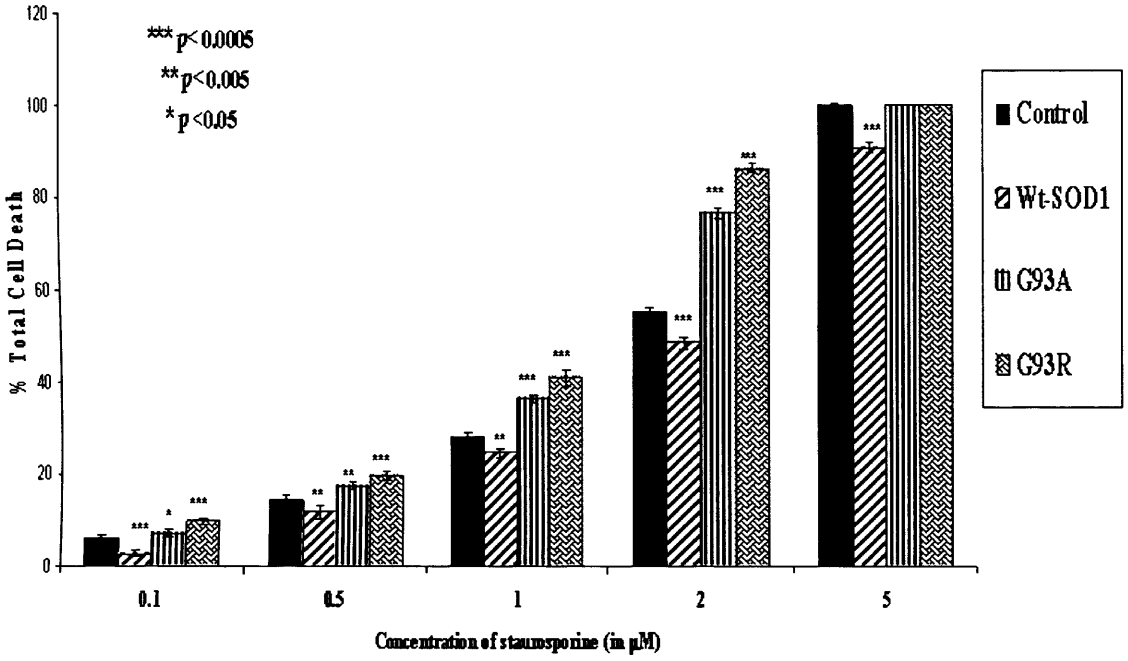



**Figure 3.4a(ii) Staurosporine Kill Curve - Effect of different concentrations of staurosporine on cell death in control, wild type or mutant SOD1 expressing cells at 4h time point.**

**a(ii).** Levels of total cell death in control vector, wt, mutant -G93A or G93R-SOD1 expressing cells exposed to 0.1µM; 0.5µM; 1µM; 2µM; 5µM staurosporine treatment for period of 4h. The proportion of cell death was assessed by trypan blue exclusion assay. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=5. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p$<0.0005). *, **, *** indicates statistically significant difference between means, when comparing with the control ND7 empty vector cell line.

Figure 3.4b

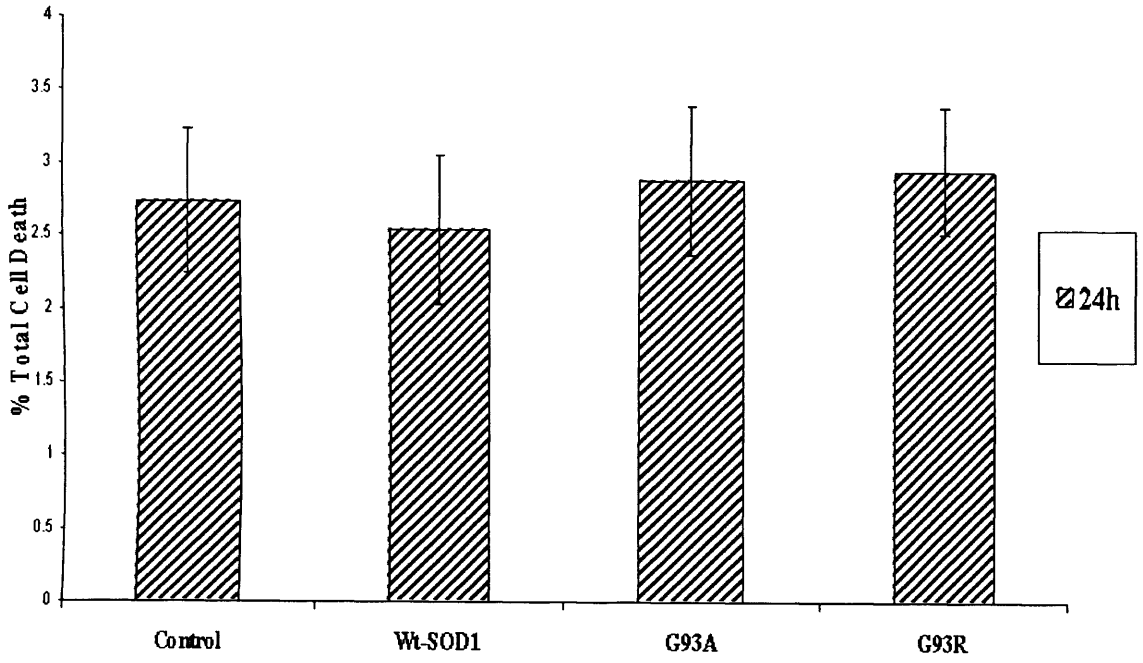



**Figure 3.4 Levels of cell death in untreated control, wild type or mutant SOD1 expressing cells at 24h.**

**b.** ND7 cell death following 24h of no treatment/no stress. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of no treatment/no stress of engineered ND7 cells expressing wt or mutant SOD1. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=5.

Figure 3.4c

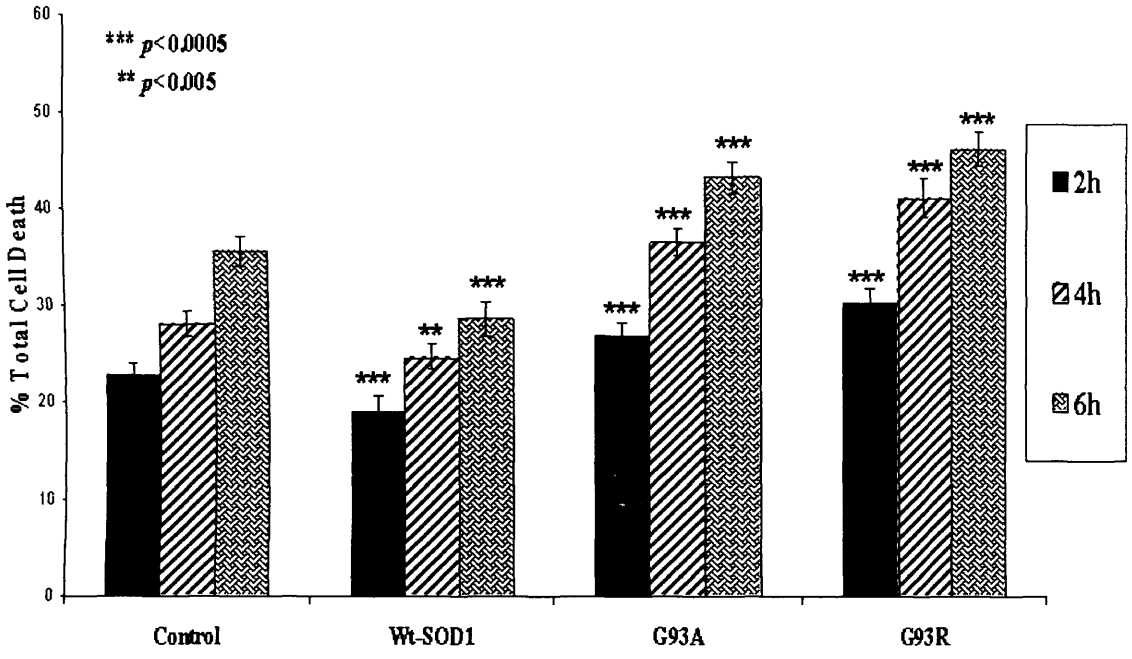



**Figure 3.4 Effect of staurosporine on cell death in control, wild type or mutant SOD1 expressing cells at different time points.**

**c.** Levels of total cell death in control vector, wt, mutant -G93A or G93R-SOD1 expressing cells exposed to staurosporine treatment for periods of 2h, 4h or 6h. The proportion of cell death was assessed by trypan blue exclusion assay. Bars represent mean $\pm$S.D. calculated for triplicate counts per sample (n), n=5. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p<0.0005$). *, **, *** indicates statistically significant difference between means, when comparing with the control ND7 empty vector cell line.

Figure 3.4d

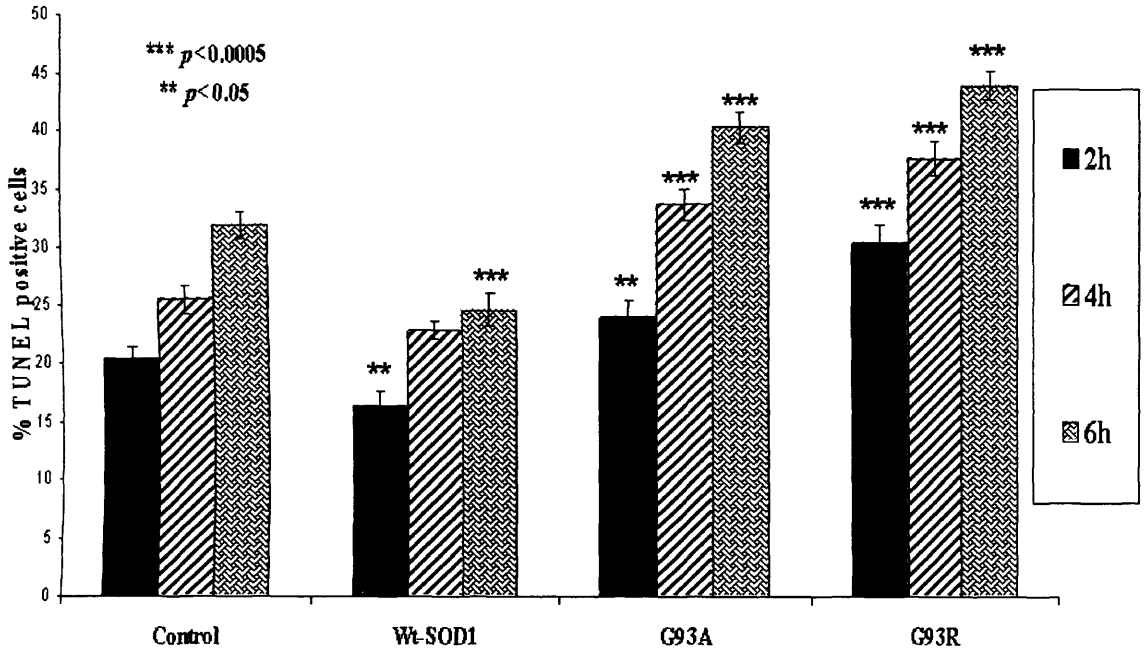



**Figure 3.4 Effect of staurosporine on cell death in control, wild type or mutant SOD1 expressing cells at different time points.**

**d.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were exposed to treatment of staurosporine for periods of 2h, 4h or 6h. Bars represent mean $^{\pm}$ S.D. calculated for triplicate counts per sample (n=3) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with the control ND7 empty vector cell line.

### 3.4.3 IFN-γ administration

Subsequently, the cells were treated with IFN-γ for 24h and 48h, which is known to induce cell death in a variety of neuronal and non-neuronal cell types (Figure 3.5a). IFN-γ proteins are found to be significantly elevated by 1.5- to 2-fold in late-stage G93A-SOD1 mouse spinal cords (Hensley et al., 2003). Therefore it was decided to investigate the effects of IFN-γ treatment further. As with serum deprivation and staurosporine, an enhanced cell death was observed in the cells over-expressing the G93R mutant and to a slightly lesser extent with the G93A mutant, whereas the cells over-expressing the wild type were significantly protected compared to the mutant cells (Figure 3.8). At the 24h and 48h time-point wt-SOD1 reduced death by approximately 23% ($p<0.0005$) and 19% ($p<0.05$) respectively compared to vector control. The G93A and G93R mutant exhibited an increase in cell death with death increasing by approximately 15% ($p<0.0005$) and 11% for G93A and 49% ($p<0.0005$) and 46% ($p<0.0005$) for G93R at 24h and 48h respectively compared to vector control.

TUNEL analysis showed a reduction in the number of TUNEL positive cells expressing wt-SOD1 with the number of TUNEL positive cells decreasing by approximately 33% and 18% at 24h and 48h respectively compared to vector control ($p<0.0005$) (Figure 3.5b). The G93A and G93R mutant expressing cells exhibited an increase in the number of TUNEL positive cells with the number of TUNEL positive cells increasing by approximately 13% at both 24h ($p<0.0005$) and 48h ($p<0.005$) for G93A and 54% ($p<0.0005$) and 56% ($p<0.0005$) for G93R at 24h and 48h respectively, compared to vector control.

Figure 3.5a

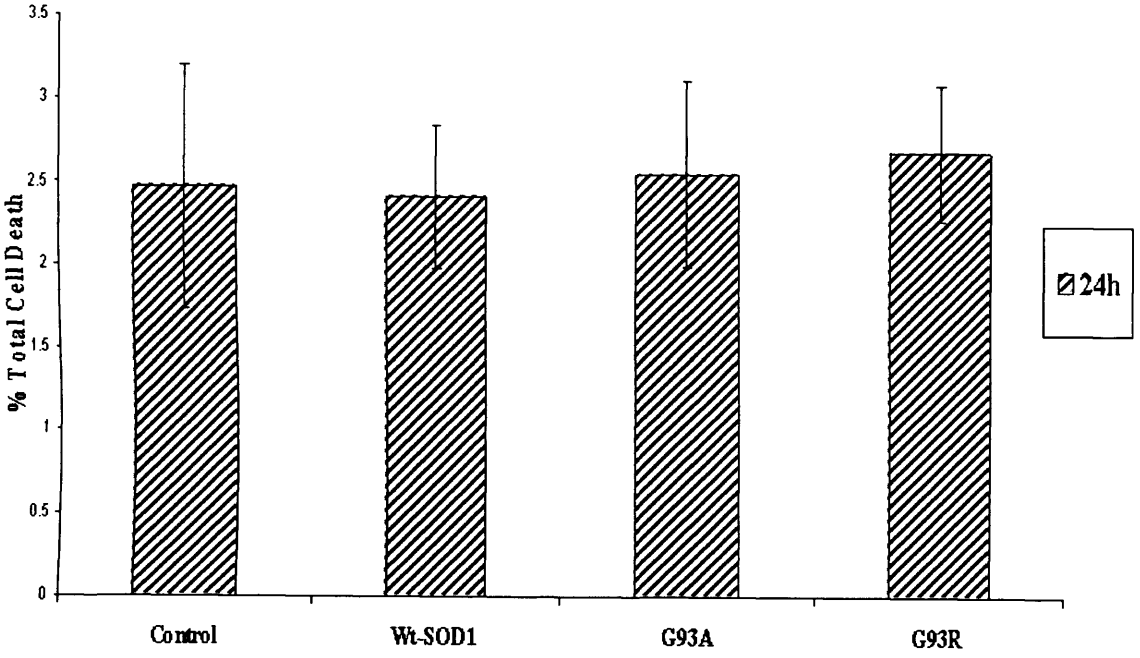



**Figure 3.5 Levels of cell death in untreated control, wild type or mutant SOD1 expressing cells at 24h.**

**a.** ND7 cell death following 24h of no treatment/no stress. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of no treatment/no stress of engineered ND7 cells expressing wt or mutant SOD1. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=5.

Figure 3.5b

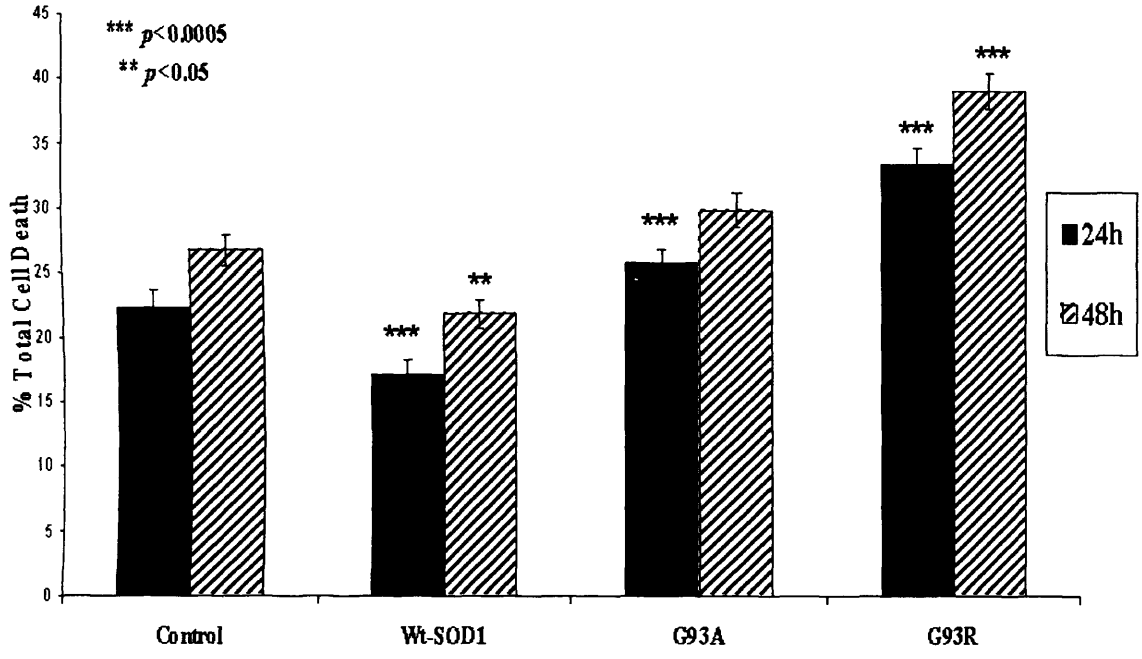



**Figure 3.5 Effect of IFN-γ on cell death in control, wild type or mutant SOD1 expressing cells at different time points.**

**b.** Levels of total cell death in control vector, wt, mutant -G93A or G93R-SOD1 expressing cells exposed to IFN-γ treatment for 24h and 48h. The proportion of cell death was assessed by trypan blue exclusion assay. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=5. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p<0.0005$). *, **, *** indicates statistically significant difference between means, when comparing with the control ND7 empty vector cell line.

Figure 3.5c

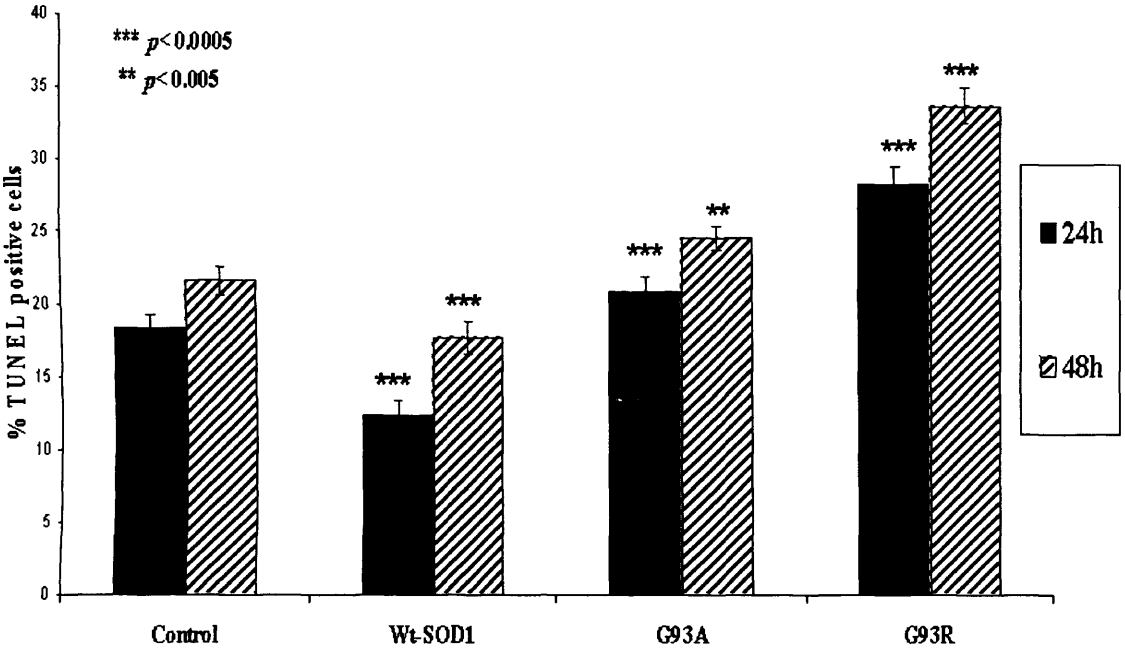



**Figure 3.5 Effect of IFN-γ on cell death in control, wild type or mutant SOD1 expressing cells at different time points.**

**c.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were exposed IFN-γ for 24h and 48h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=3) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with the control ND7 empty vector cell line.

### 3.4.4 Hydrogen Peroxide ($H_2O_2$) treatment

Altered reactivity for certain substrates has been observed for mutant SOD1 enzyme. Dismutase activity of FALS-associated SOD1 mutations is normal but peroxidase activity appears to be enhanced (Wiedau-Pazos et al., 1996; Yim et al., 1996). X-ray crystallographic studies have revealed that the active channel of FALS mutant is slightly larger than that of the wild type, providing support to the theory that $H_2O_2$ has access to the mutant enzyme (Yim et al., 1996). Hydrogen peroxide ($H_2O_2$) is well known to inactivate Cu, Zn SOD through the oxidation of a histidine ligand for copper. The reaction is thought to be a two-step process, with the anion of hydrogen peroxide ($HOO^-$) first reducing $Cu^{2+}$ to $Cu^{1+}$. The reduced copper is then thought to react with a second hydrogen peroxide to produce a strong oxidant with the reactivity of a hydroxyl radical that has a high probability of then attacking a nearby histidine ligand. Therefore it was decided to investigate the effects of $H_2O_2$ treatment further in the SOD1 expressing stable cells.

G93A and G93R mutant SOD1 over-expressing cells exhibited an increase in total cell death of approximately 10% ($p<0.05$) and 44% ($p<0.0005$) respectively at the 24h time point compared to control (Figure 3.6a). At the 48h time point the cell death increased to approximately 25% and 51% for G93A and G93R respectively compared to control ($p<0.0005$). Wt SOD1 cells were protected against the insult with cell death being reduced by approximately 16% and 26% at 24h and 48h respectively compared to control ($p<0.0005$).

TUNEL analysis further supported the cell death results with the number of TUNEL positive cells for the G93A and G93R mutant increasing by approximately 16% ($p<0.05$) and 52% ($p<0.0005$) respectively at 24h and 29% ($p<0.0005$) and 55% ($p<0.0005$) respectively at 48h compared to control (Figure 3.6b). Wt-SOD1 cells exhibited a reduction in the number of TUNEL positive cells with number of TUNEL positive cell decreasing by approximately 16% and 28% ($p<0.0005$) at 24h and 48h respectively compared to control.

Figure 3.6a

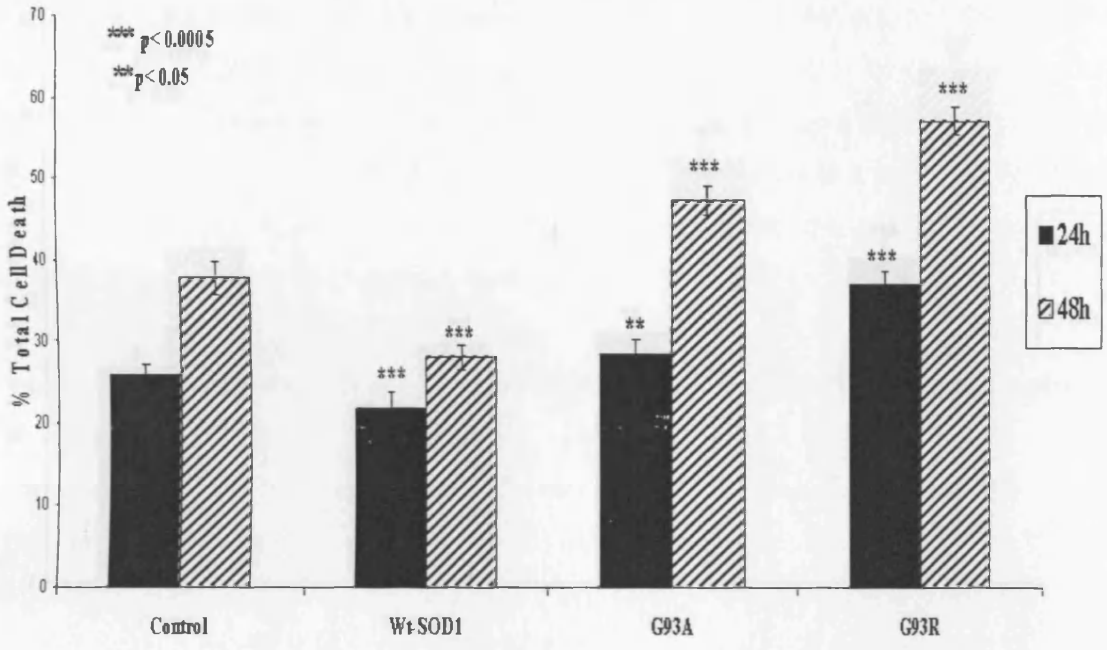



**Figure 3.6 Effect of 10μM $H_2O_2$ on cell death in control, wild type or mutant SOD1 expressing cells at different time points.**

**a.** Levels of total cell death in control vector, wt, mutant -G93A or G93R-SOD1 expressing cells exposed to $H_2O_2$ treatment for periods of 24h and 48h. The proportion of cell death was assessed by trypan blue exclusion assay. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=5. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p$<0.0005). *, **, *** indicates statistically significant difference between means, when comparing with the control ND7 empty vector cell line.

Figure 3.6b

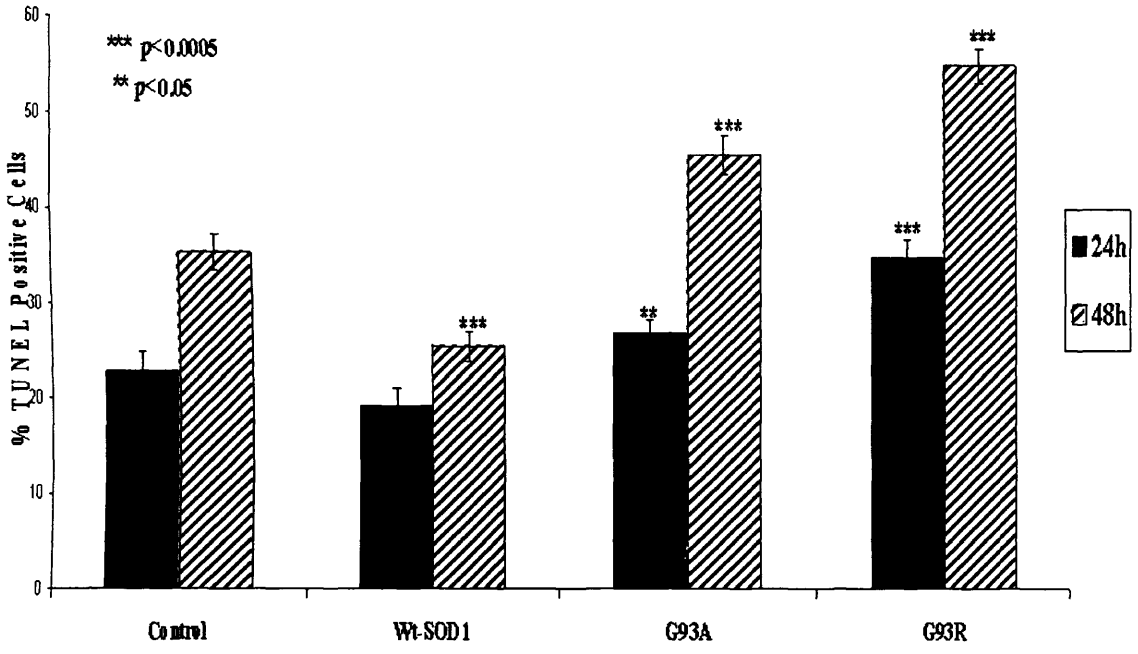



**Figure 3.6 Effect of 10μM $H_2O_2$ on cell death in control, wild type or mutant SOD1 expressing cells at different time points.**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were exposed to treatment of $H_2O_2$ for periods of 24h and 48h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=3) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with the control ND7 empty vector cell line.

### 3.4.5 Glutamate administration

Besides oxidative stress, glutamate-mediated excitotoxicity is considered to be an important factor of motor neuron degeneration observed in ALS. In fact, glutamate accumulation into the synaptic cleft results in excessive post-synaptic receptor stimulation, calcium entry and cell death (Choi, 1988; Beal, 1992; Coyle and Puttfarcken, 1993). Consistently, in ALS, there is evidence that a defect in glutamate turnover leads to increased levels of extracellular glutamate with resulting deleterious consequences (Atlante et al., 2001; Leigh and Meldrum, 1996; Doble 1999). Therefore it was decided to investigate the effects of glutamate administration in the SOD1 expressing stable cells.

Mutant SOD1 expressing cells exhibited an increase in total cell death with death increasing by approximately 6% and 46% ($p<0.0005$) for G93A and G93R respectively at 24h compared to control (Figure 3.7a). At the 48h time point the G93A and G93R mutant exhibited an approximately 21% ($p<0.0005$) and 63% ($p<0.0005$) increase in cell death respectively compared to control. Wt-SOD1 expressing cells provided a protective effect with cell death reduced by approximately 23% ($p<0.0005$) and 10% ($p<0.05$) at 24h and 48h respectively compared to control.

TUNEL analysis supported the total cell death results. At the 24h time point G93A and G93R expressing mutant cells exhibited an increase in the number of TUNEL positive cells with the number of TUNEL positive cells increasing by approximately 10% ($p<0.05$) and 54% ($p<0.0005$) respectively compared to control (Figure 3.7b). At the 48h time point G93A and G93R mutant cells exhibited an approximately 21% ($p<0.05$) and 62% ($p<0.0005$) increase in the number of TUNEL positive cells respectively compared to control. Wt-SOD1 cells exhibited an approximately 27% ($p<0.0005$) and 17% ($p<0.01$) reduction in the number of TUNEL positive cells at 24h and 48h respectively compared to control.

Figure 3.7a

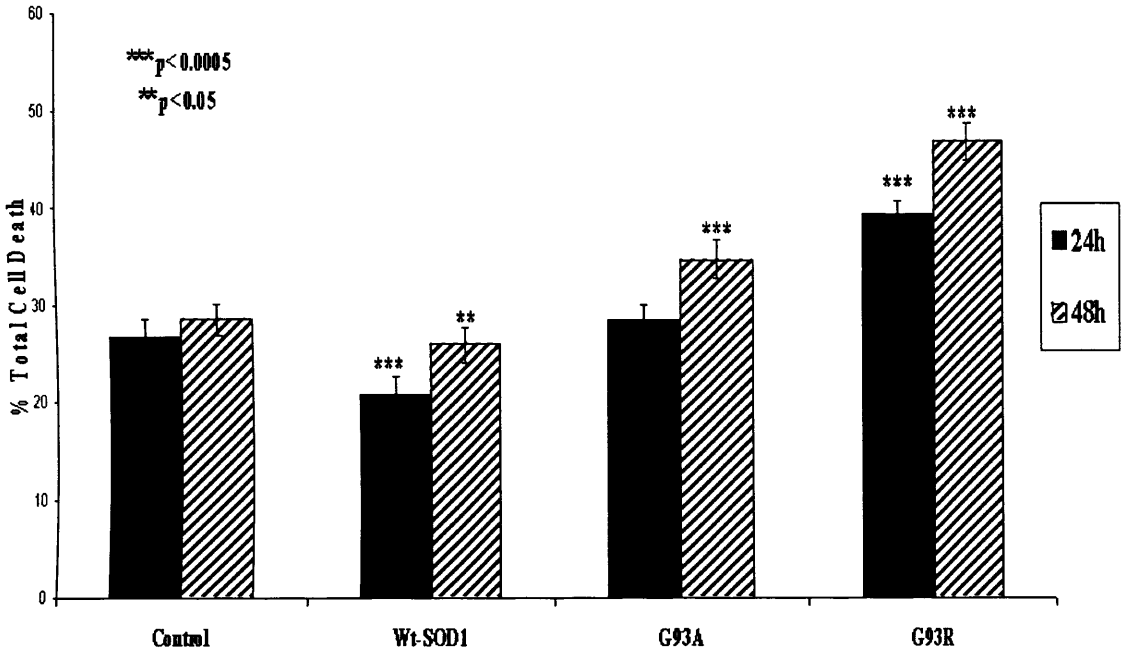



**Figure 3.7 Effect of 5μM Glutamate on cell death in control, wild type or mutant SOD1 expressing cells at different time points.**

**a.** Levels of total cell death in control vector, wt, mutant -G93A or G93R-SOD1 expressing cells exposed to Glutamate treatment for periods of 24h and 48h. The proportion of cell death was assessed by trypan blue exclusion assay. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=5. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p$<0.0005). *, **, *** indicates statistically significant difference between means, when comparing with the control ND7 empty vector cell line.

Figure 3.7b

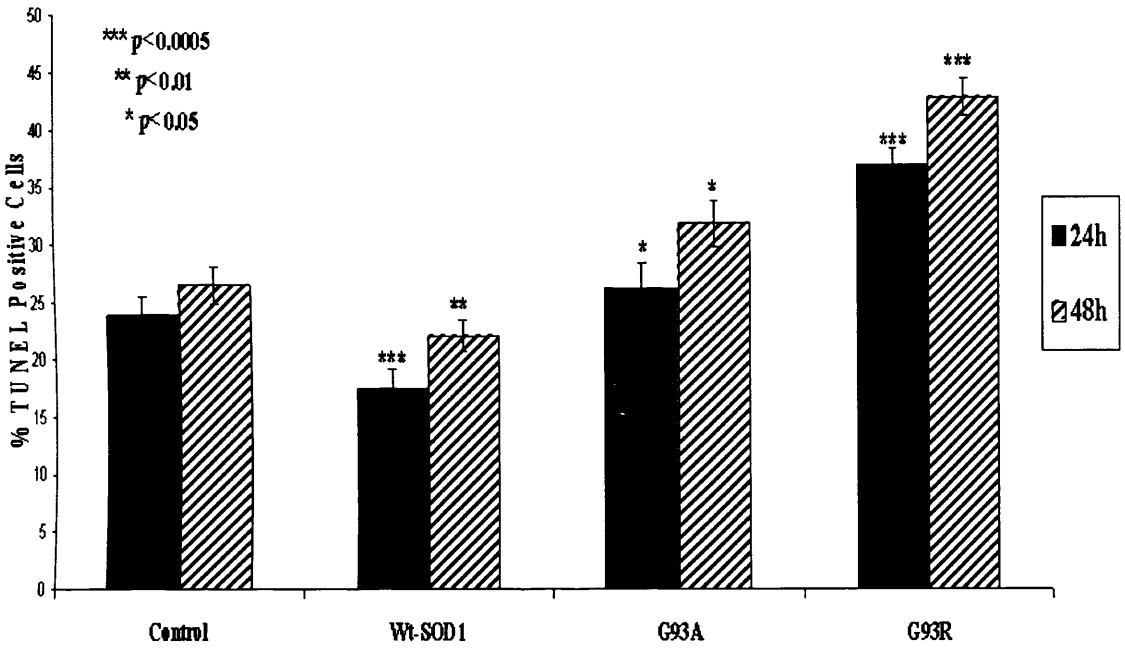



**Figure 3.7 Effect of 5μM Glutamate on cell death in control, wild type or mutant SOD1 expressing cells at different time points.**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were exposed to treatment of Glutamate for periods of 24h and 48h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=3) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with the control ND7 empty vector cell line.

### 3.4.6 Camptothecin administration

Similar results were also observed when the cells were treated with camptothecin (Figure 3.8a). Camptothecin works by targeting topoisomerase I, inducing single strand breaks, thereby affecting the cells capacity to replicate and eventually leading to apoptotic death mediated by caspase activation. Wt-SOD1 expressing cells exhibited a decrease in cell death with death decreasing by approximately 24% and 21% at 24h and 48h respectively, compared to vector control ($p<0.0005$). The mutants once again exhibited an increase in cell death with death increasing by approximately 25% and 27% for G93A at 24h and 48h respectively ($p<0.0005$). The G93R mutant showed a larger increase in death in line with previous results with death increasing by 43% at both 24h and 48h respectively compared to vector control ($p<0.0005$).

TUNEL analysis revealed a reduction in the number of TUNEL positive cells in the cells expressing wt-SOD1 with the number of TUNEL positive cells being reduced by approximately 30% and 31% at 24h and 48h respectively compared to vector control ($p<0.0005$) (Figure 3.8b). The mutants on the other hand exhibited an increase in the number of TUNEL positive cells with the number increasing by approximately 29% ($p<0.0005$) and 7% ($p<0.05$) for G93A at 24h and 48h respectively, compared to vector control. The G93R mutant once again exhibited a much larger increase in TUNEL positive cells with number increasing by approximately 53% and 39% at 24h and 48h respectively compared to vector control ($p<0.0005$).

Figure 3.8a

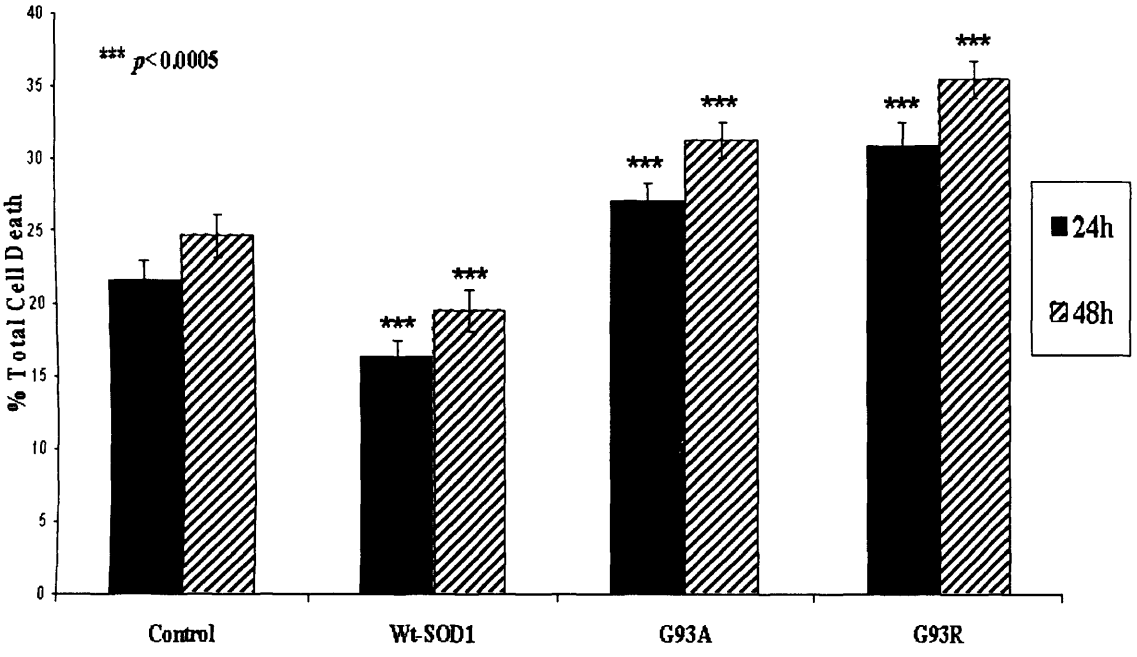



**Figure 3.8 Effect of Camptothecin on cell death in control, wild type or mutant SOD1 expressing cells at different time points.**

**a.** Levels of total cell death in control vector, wt, mutant -G93A or G93R-SOD1 expressing cells exposed to Camptothecin treatment for periods of 24h and 48h. The proportion of cell death was assessed by trypan blue exclusion assay. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=5. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p$<0.0005). *, **, *** indicates statistically significant difference between means, when comparing with the control ND7 empty vector cell line.

Figure 3.8b

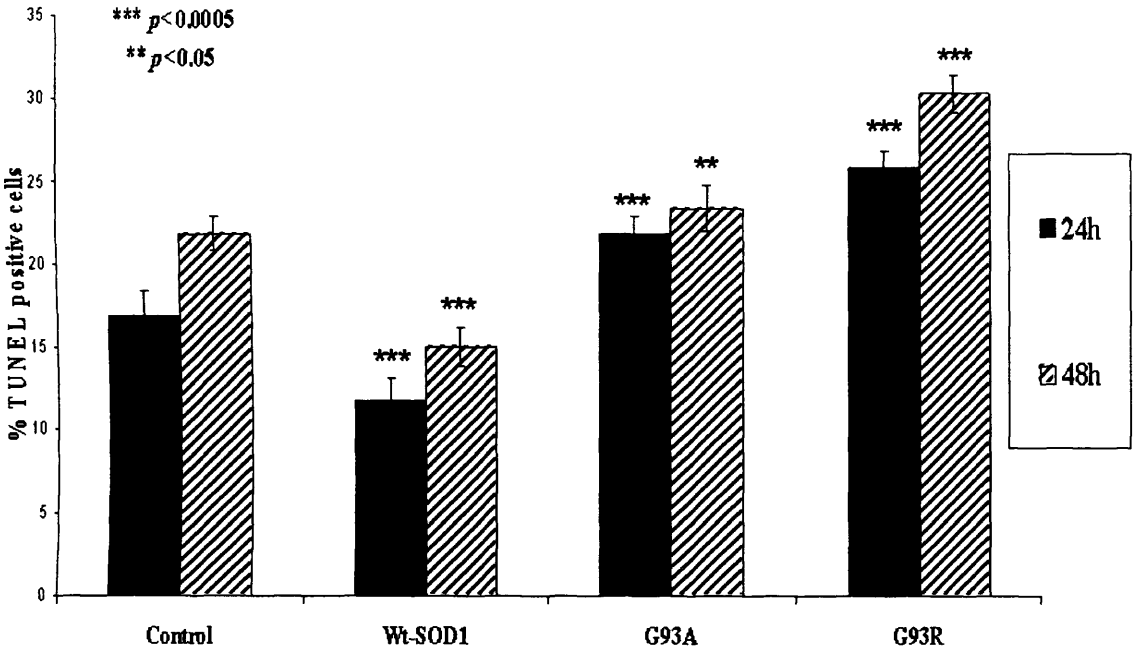



**Figure 3.8 Effect of Camptothecin on cell death in control, wild type or mutant SOD1 expressing cells at different time points.**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were exposed to treatment of Camptothecin for periods of 24h and 48h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=3) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with the control ND7 empty vector cell line.

### 3.4.7 Cell - line responses to Simulated Ischemia/Reoxygenation.

In view of the role of SOD as an antioxidant and in dealing with free radicals formed in cells, it was of particular interest to investigate the response of these cells to a period of simulated ischemia followed by reoxygenation which has been used in the past to induce cell death in ND7 cells and also to demonstrate the neuroprotective role of Hsps (Wagstaff et al., 1999). Briefly, the cells were plated in 6-well plates and subjected to ischemia for a period of 1h, 2h, 3h or 4h followed by 24h of reoxygenation. The control buffer (pH 7.4) was used at all times and the cell death observed was between 5-10% in the various cell lines (data not displayed). It is clear, however, that the cytotoxic effect observed of the ischemic buffer is because of its additional components and the reduced pH.

An increase in death was observed in all the cell lines on ischemia/reperfusion over ischemia alone. The greater the period of ischemia the greater was the death observed and the greater the corresponding death seen on reoxygenation. The deleterious effects of the mutants were more clearly observed on reoxygenation. The results of this experiment (Figure 3.9) paralleled those obtained for the other stimuli, indicating over-expression of both the mutants significantly enhanced the degree of cell death observed, with the G93R mutation having a stronger and more dramatic effect than the G93A mutation. However, cells transfected with the wild type SOD1 showed significantly reduced cell death compared to the cells expressing either of the two mutants corroborating that the mutants had a particularly damaging effect in the response to simulated ischemia/reoxygenation as with the other stimuli. G93A mutant cells exhibited an approximately 11%, 14% and 27% ($p<0.0005$) increase in total cell death following 1h, 2h and 3h of ischemia respectively compared to ND7 empty vector control (Figure 3.9a). G93R mutant cells showed a larger increase with death increasing by approximately 18%, 42% ($p<0.0005$) and 55% ($p<0.0005$) following 1h, 2h and 3h periods of ischemia respectively compared to ND7 empty vector control (Figure 3.9a). Wt-SOD1 cells exhibited a protective effect with death being reduced by approximately 29% ($p<0.05$), 26% ($p<0.005$) and 26% ($p<0.0005$) following 1h, 2h and 3h periods of ischemia respectively compared to ND7 empty vector control (Figure 3.9a). A larger increase in death was observed on reoxygenation. G93A cells exhibited an increase in cell death by approximately 18% ($p<0.005$), 13% and 16% ($p<0.005$) at 1h, 2h and 3h of

ischemia followed by 24h of reoxygenation respectively compared to control (Figure 3.9a). G93R cells exhibited a more dramatic increase in cell death with death increasing by approximately 60%, 72% and 87% following 1h, 2h and 3h of ischemia followed by 24h of reperfusion compared to control ($p<0.0005$) (Figure 3.9a). Wt-SOD1 cells exhibited a reduction in cell death with death being reduced by approximately 31%, 32% and 27% after 1h, 2h and 3h of ischemia followed by 24h of reoxygenation compared to control ($p<0.0005$).

TUNEL analysis revealed similar results with the number of TUNEL positive cells of both mutants increasing following increasing periods of ischemia and increasing further on the corresponding reoxygenation compared to control ($p<0.0005$) (Figure 3.9b). Wt-SOD1 cells exhibited a protective effect with the number of TUNEL positive cells being reduced at each instance compared to ND7 empty vector control.

Figure 3.9a

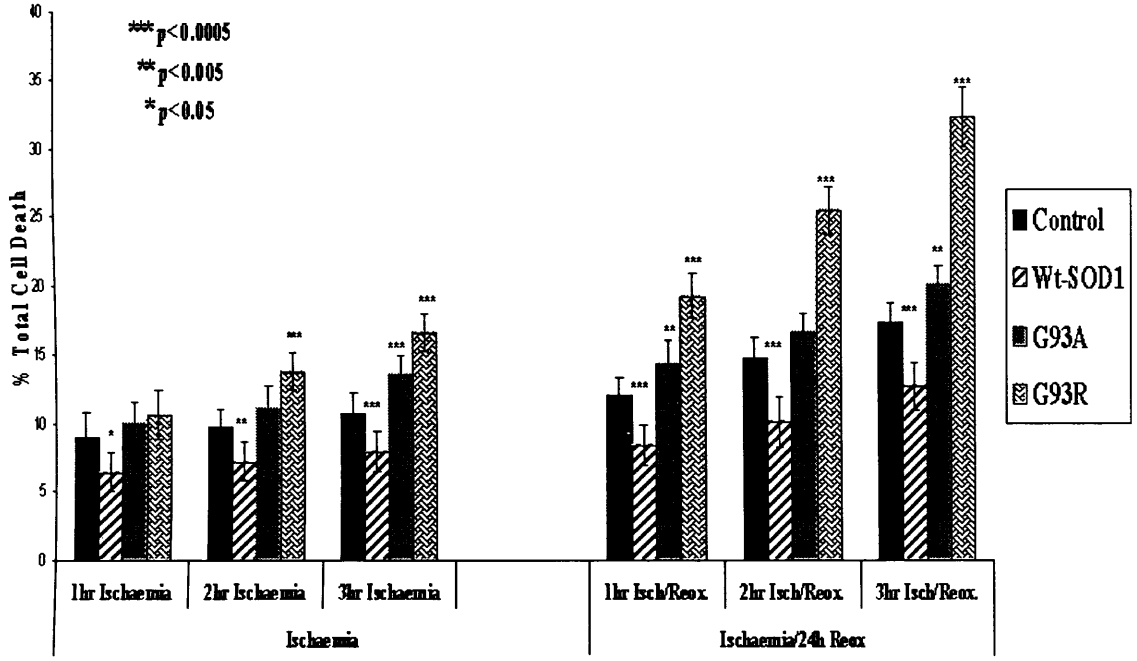



**Figure 3.9 Effect of simulated ischemia and ischemia followed by 24h of reoxygenation on cell death in control, wild type or mutant SOD1 expressing cells at different time points.**

**a.** Levels of total cell death in control vector, wt, mutant -G93A or G93R-SOD1 expressing cells exposed to 1h, 2h or 3h of ischemia and 1h, 2h or 3h of ischemia followed by 24h of reoxygenation. The proportion of cell death was assessed by trypan blue exclusion assay. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=5. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p$<0.0005). *, **, *** indicates statistically significant difference between means, when comparing with the control ND7 empty vector cell line.

Figure 3.9b

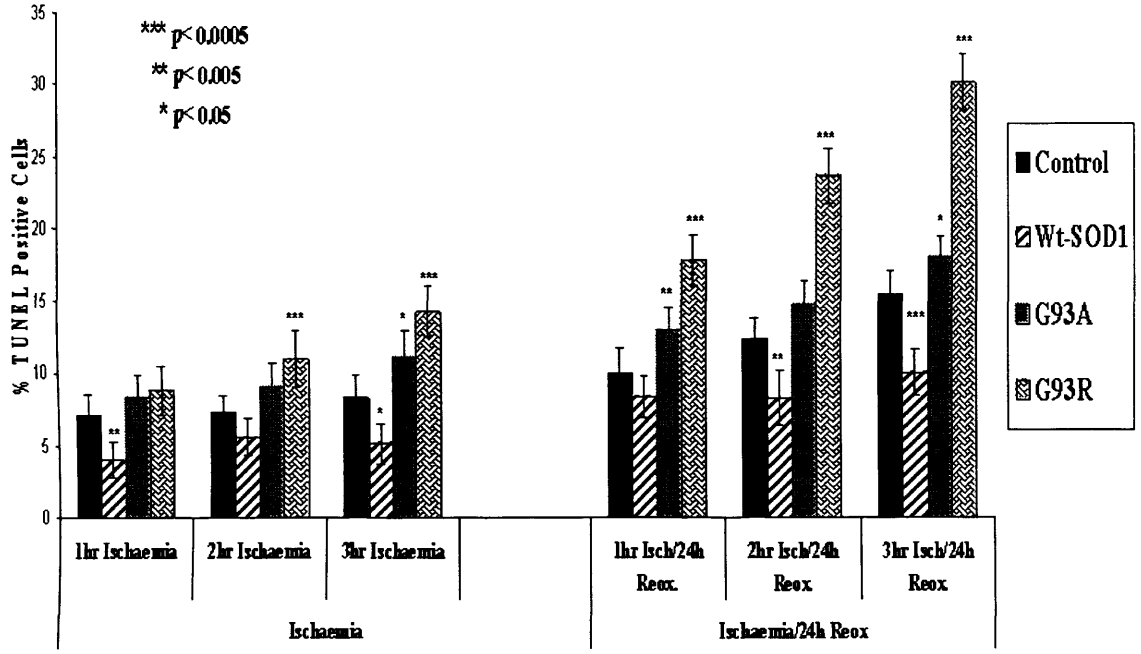



**Figure 3.9 Effect of simulated ischemia and ischemia followed by 24h of reoxygenation on cell death in control, wild type or mutant SOD1 expressing cells at different time points.**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were exposed to treatment of 1h, 2h or 3h of ischemia 1h, 2h or 3h of ischemia followed by 24h of reoxygenation. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=5) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with the control ND7 empty vector cell line.

### 3.4.8 4hr Ischemia plus 24h of Reoxygenation

Wt-SOD1 expressing cells exposed to 4hr ischemia+24h reoxygenation exhibited a reduction in death of approximately 19% compared to vector control ($p<0.01$); (Figure 3.10a). The G93A and G93R mutant expressing cells exhibited an increase in death of approximately 13% and 86% respectively compared to vector control ($p<0.0005$) (Figure 3.10a).

TUNEL analysis revealed a similar trend with the number of TUNEL positive cells for wt-SOD1 cell line being reduced by approximately 24% compared to control (Figure 3.10b). The G93A and G93R mutant expressing cells exhibited an increase in the number of TUNEL positive cells with number increasing by approximately 10% and 100% ($p<0.0005$) respectively compared to vector control (Figure 3.10b).

Figure 3.10a

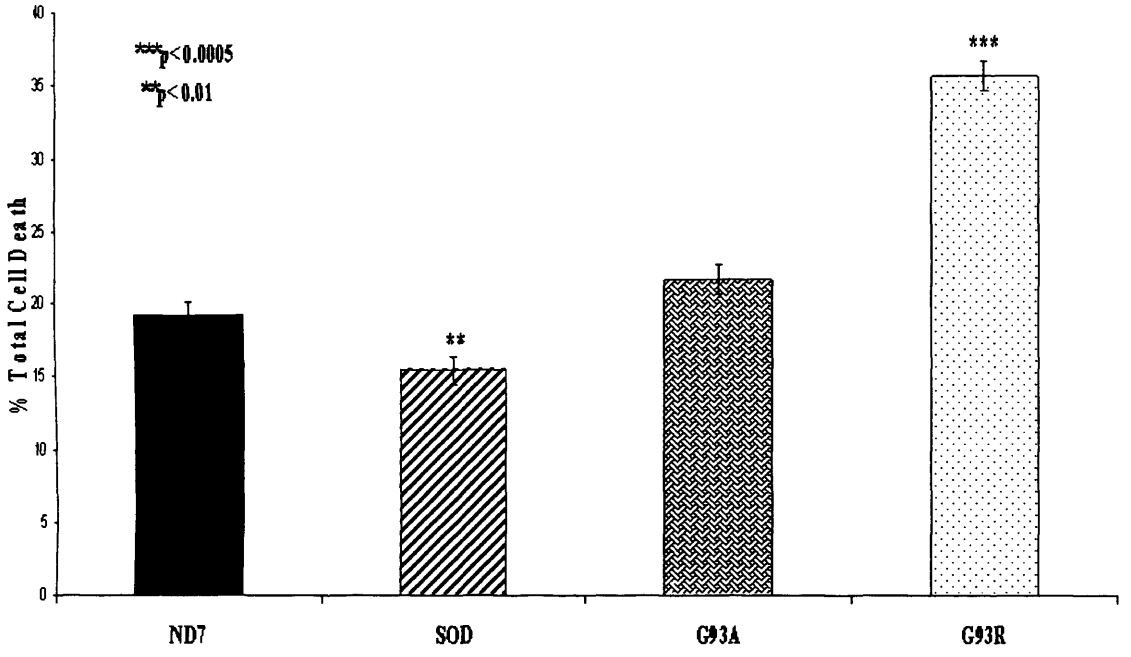



**Figure 3.10 Effect of 4h of simulated ischemia followed by reoxygenation on cell death of ND7 cells expressing wild type or mutant SOD1.**

**a.** Control, wt and mutant SOD1 cell lines were exposed to 4h of ischemia followed by 24h of reoxygenation. Cell death was immediately assessed by the trypan blue assay. The proportion of cell death was assessed by trypan blue exclusion assay. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=6. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p<0.0005$). *, **, *** indicates statistically significant difference between means, when comparing with the control ND7 empty vector cell line.

Figure 3.10b

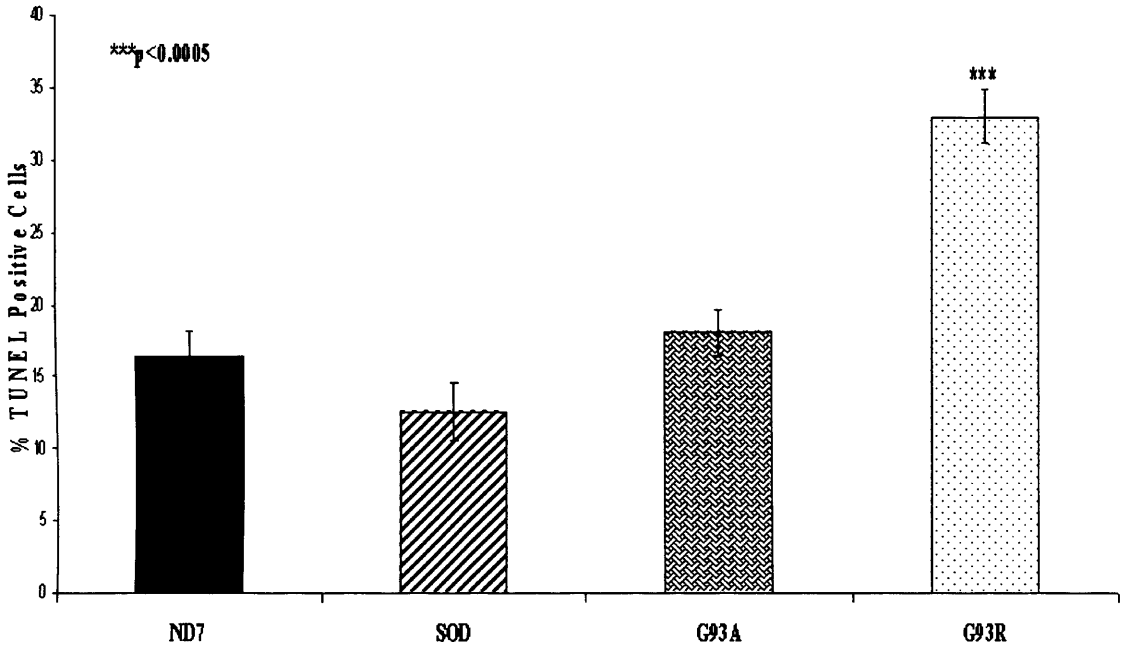



**Figure 3.10 Effect of 4h of simulated ischemia followed by reoxygenation on cell death of ND7 cells expressing wild type or mutant SOD1.**

**b.** Percentage of TUNEL-positive cells counted in three different fields in six experiments where cell lines were exposed to treatment of 4h of ischemia followed by 24h of reoxygenation. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=6) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with the control ND7 empty vector cell line.

### 3.4.9 4h of Ischemia followed by 24h of Reoxygenation plus Retinoic Acid (RA)

Addition of differing concentrations of retinoic acid to the cell lines, 2 hours prior to being subjected to 4hr of ischemia followed by 24h of reoxygenation, produced interesting results. In contrast to the effect of RA when serum was removed, addition of retinoic acid under these conditions produced an initial protective effect in all cells with the protective effect lasting to varying extents across the cells. Wt-SOD1 cells exhibited the greatest protection on addition of 1μM retinoic acid with death reduced by approximately 22% compared to control ($p<0.005$); (Figure 3.11a). The G93A mutant exhibited the greatest protection at 2μM concentration of retinoic acid with death reduced by approximately 58% compared to control ($p<0.0005$). The G93R mutant exhibited the greatest reduction in death at a slightly higher concentration with death reduced by approximately 72% compared to control, on addition of 4μM retinoic acid ($p<0.0005$). The protection provided to the cell lines by differing concentrations of retinoic acid can be easily seen in Figure 3.11b.

TUNEL analysis paralleled trypan blue results with initial protection observed across all cells on addition of retinoic acid (Figure 3.11c). Wt-SOD1 cells exhibited a 20% reduction in the number of TUNEL positive cells on addition of 1μM retinoic acid compared to control ($p<0.05$); (Figure 3.11c). The G93A mutant exhibited an approximately 60% reduction in the number of TUNEL positive cells at 2μM concentration of retinoic acid compared to control ($p<0.0005$) whereas the G93R mutant exhibited an approximately 76% reduction in the number of TUNEL positive cells on addition of 4μM retinoic acid compared to control ($p<0.0005$).

Figure 3.11a

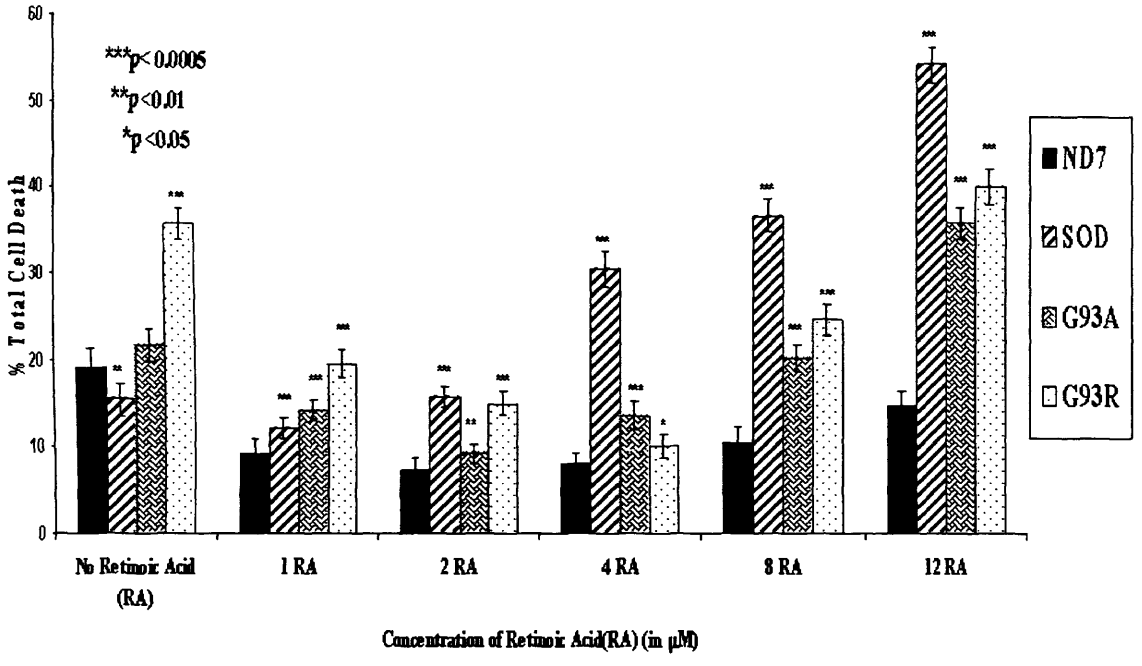



**Figure 3.11a Effect of 4h of ischemia followed by 24h of reoxygenation and varying concentrations of retinoic acid on cell death in control, wild type or mutant SOD1 expressing cells.**

Levels of total cell death in control vector, wt, mutant -G93A or G93R-SOD1 expressing cells exposed to 4h of ischemia followed by 24h of reoxygenation plus varying concentrations of retinoic acid. The proportion of cell death was assessed by trypan blue exclusion assay. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=6. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p$<0.0005). *, **, *** indicates statistically significant difference between means, when comparing with the control ND7 empty vector cell line.

Figure 3.11b

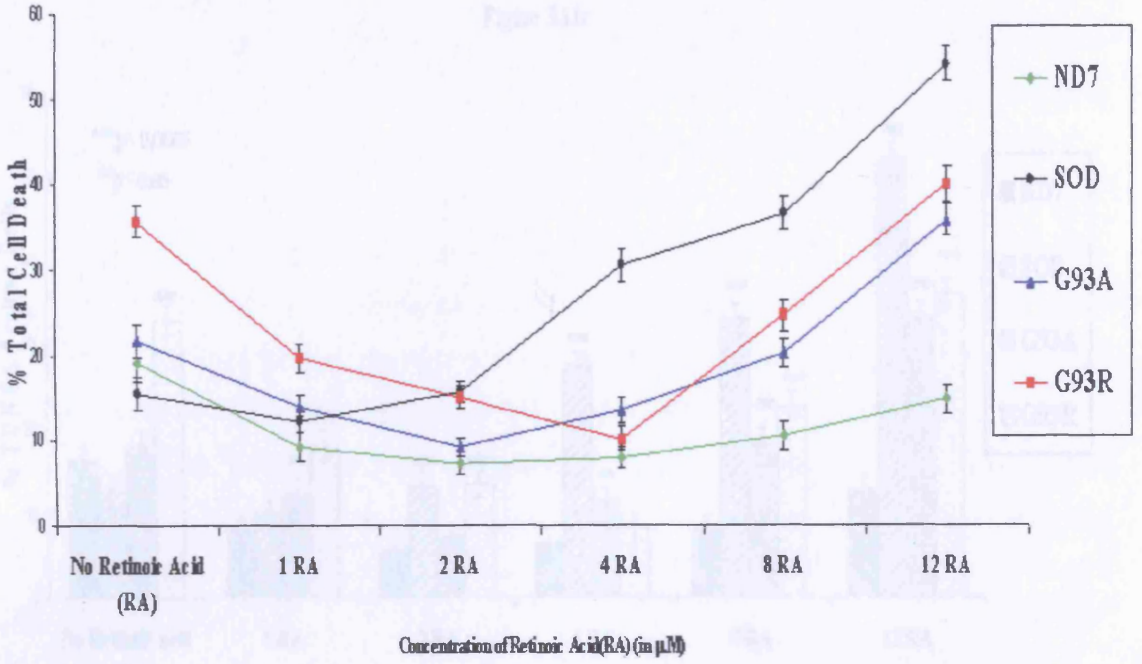



**Figure 3.11b Effect of 4h of ischemia followed by 24h of reoxygenation and varying concentrations of retinoic acid on cell death in control, wild type or mutant SOD1 expressing cells.**

Line graph showing levels of total cell death in control vector, wt, mutant -G93A or G93R-SOD1 expressing cells exposed to 4h of ischemia followed by 24h of reoxygenation plus varying concentrations of retinoic acid. The proportion of cell death was assessed by trypan blue exclusion assay.

Figure 3.11c

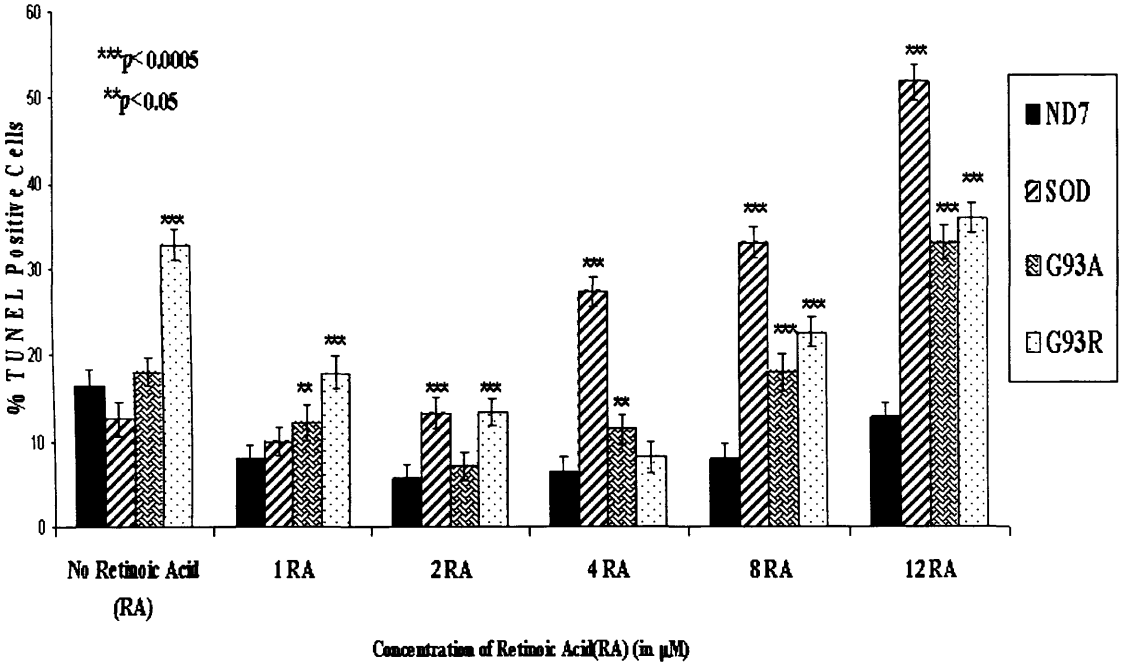



**Figure 3.11c Effect of 4h of ischemia followed by 24h of reoxygenation and varying concentrations of retinoic acid on cell death in control, wild type or mutant SOD1 expressing cells.**

Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were exposed to treatment of 4h of ischemia followed by 24h of reoxygenation plus varying concentrations of retinoic acid. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=6) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with the control ND7 empty vector cell line.

## 3.5 Discussion

The findings presented in this chapter describe the establishment and characterisation of an *in vitro* model system where wt-SOD1 and the disease-associated SOD1-mutant forms are over-expressed. It was shown that the response of the cell lines to various death-inducing stimuli depends on both the type of stress and the form of SOD1, which is over-expressed.

There was a statistically significant reduction in cell death in ND7 cells over-expressing human wt-SOD1 compared to control cells (stably transfected with the vector alone) when cell lines were exposed to wide-range of stresses including serum withdrawal, serum withdrawal in the presence of retinoic acid, IFN-γ, staurosporine, camptothecin, hydrogen peroxide, glutamate, and ischemia/reoxygenation. In the case of the two mutant forms of SOD1, cell death was always enhanced compared to control cells with the differences in cell death between control and mutant cell lines being statistically significant for all the stresses tested.

The above findings suggest a deleterious function for both the mutant forms of the protein. When the cell lines expressing the two mutant forms of SOD1 were compared against each together, G93R mutant exhibited an enhanced cell death over the G93A mutant throughout all the stresses tested. In general, the G93A mutant exhibited a slightly higher death than control cells under all stresses tested. The difference in cell death between the two mutant forms may be the result of a number of different factors such as differing degrees of copper binding, differences in zinc affinity, or even differences in size of active site. Overall, the above data provides support to the proposed hypothesis that the disease-associated FALS SOD1 mutations confer a gain of function and /or loss of a protective function on this protein, resulting in the deleterious effects observed in the cells and that wt-SOD1 protein provides a uniformly protective effect on the cells against all stresses tested.

The stresses tested have been widely used by others and are relevant to ALS in that serum deprivation simulates deprivation of survival signals to neurons, glutamate induced excitotoxicity as well as hydrogen peroxide induce oxidative stress which is also relevant to the neuronal environment. IFN-γ protein is significantly elevated 1.5- to 2-fold in late-stage

G93A-SOD1 mouse spinal cords (Hensley et al., 2003) and is therefore a relevant stress. Camptothecin works by targeting topoisomerase I, inducing single strand breaks, thereby affecting the cells capacity to replicate and eventually resulting in apoptotic death mediated by caspase activation. The use of simulated ischemia has not been previously been reported but is a physiological stress that was used to challenge ND7 cells and induce cell death, thereby enabling assessment of the effect of SOD1 on ND7 cell survival (Wagstaff et al., 1999). Staurosporine is a well-studied apoptotic agent, a bacterial alkaloid that inhibits several cellular kinases (Gill et al., 2003) and is frequently used as an inducer of the mitochondrial apoptotic pathway. Intracellular cascades activated by staurosporine depend on its concentration, with 100nM concentration inducing cell death by means of an apoptotic mechanism characterised by cytochrome *c* release and a 1μM concentration of staurosporine inducing cell death through a mechanism, in which caspase activation is detected, but which shows several nonapoptotic features (Deshmukh et al., 2000); both apoptotic and nonapoptotic features have been described in ALS (reviewed in Chapter 1, Introduction). Since apoptosis and also non-apoptotic cell death are known to be implicated in neurodegeneration (reviewed in Chapter 1, Introduction), all the stresses utilized in this study have been previously demonstrated to induce programmed cell death in ND7 cells or other neuronal cell lines and hence were chosen for this project.

As mentioned in the introduction of this chapter, SOD1 is an anti-apoptotic protein and has been intensively investigated due to its biological importance as an antioxidant; there exists a substantial amount of evidence which continues to accumulate that supports the idea that the mutations appearing in the familial cases of ALS are deleterious in most model systems studied. The system described in this chapter utilized a well characterized neuronal cell line in which the exogenous expression of SOD1 mutants has a differential effect on neuronal cell survival, depending on the mutant under consideration, when subjected to various cellular stresses that result predominantly in apoptotic cell death.

The findings presented here, suggest that the disease-associated SOD1 mutants G93A and G93R enhance the level of cell death observed in a neuronal cell line exposed to a variety of apoptotic stimuli such as serum removal, staurosporine treatment, γ-interferon or exposure to simulated ischemia followed by reoxygenation. The results of the present study

extends the work of Rabizadeh et al., (1995) who demonstrated that the SOD1 mutations G37R and A4V associated with ALS converted the anti-apoptotic SOD1 gene into a pro-apoptotic gene. Furthermore, in the present study the G93R mutation was observed to be a lot more severe and exhibited enhanced levels of death compared to the G93A mutation, across all the stresses investigated, paralleling the much more severe disease phenotype and early onset observed in ALS patients with this mutation. Even though the G93R mutation has only been reported in a single family (de Belleroche et al., 1995; Orrell et al., 1995 & 1997), it is associated with a particularly early age of onset of 36 years (4 individuals with a range of age of onset of 29-41 years).

Several studies previously demonstrated a protective effect of the wild type SOD1 against neuronal apoptosis (Greenlund et al., 1995; Rabizadeh et al., 1995) and this has also been shown to be the case in the present experimental system. Thus, the wild type SOD1 protein possesses the ability to protect the neuronal cell line against a wide variety of apoptotic stresses including serum removal both in the absence and presence of 1μM retinoic acid, staurosporine administration, IFN-γ treatment, camptothecin addition, glutamate administration, hydrogen peroxide treatment and ischemia/reoxygenation. Hence, in a wide range of different situations the wild type SOD1 is protective whilst the G93A or G93R mutations in some way convert the protein from an anti-apoptotic into a pro-apoptotic form. This is in contrast to our findings with Parkinson's disease-associated mutations of α-synuclein and the wild-type protein (Zourlidou et al., 2003). Zourlidou et al. (2003) have previously demonstrated that the two-disease associated mutations of α-synuclein result in enhanced sensitivity to serum removal or simulated ischemia when they are over-expressed in ND7 cells. In contrast however, overexpression of the wild-type α-synuclein results in a protective effect against serum removal but produces a damaging effect, albeit less than observed with the mutants, when exposed to simulated ischemia. Hence, in the case of α-synuclein the wild-type protein appears to produce a protective effect against some stresses but not others, whereas the Parkinson's disease-associated mutations have a pro-apoptotic effect in cells exposed to a wide-variety of different stresses.

In this Chapter it was shown that disease-associated mutants of SOD1 enhance the degree of cell death observed in response to a wide variety of different stimuli with the

severity of this effect paralleling the severity of the disease caused by the specific mutation in question. In contrast, over-expression of wild-type SOD1 has an anti-apoptotic effect protecting cells from a wide range of death-inducing stimuli. One potential problem that arises with all experiments involving stable cell lines is variation in both the levels of expression of the transgene and the final phenotype of the different clones, both phenomena probably being a result of site of integration of DNA sequence of the transgene within the cell genome. However, this is easily countered by using several different cell clones with each plasmid.

In addition, in this study endogenous levels of SOD1 and levels of overexpression of SOD1 were not determined. It would be of interest to know the levels of endogenous SOD1 and the level of overexpression of SOD1 so that one can ascertain the real extent of the findings presented here and also to ensure the levels are not such that the biology of the cell itself is altered.

Another limitation of this system is the fact that ND7 cells are not primary cells, which would be more relevant to the disease associated mutations, although there are numerous studies describing interesting effects with SOD1 and its mutant forms in non-neuronal as well as non-mammalian systems (for review see Chapter 1). ND7 cells are derived from a fusion of rat dorsal root ganglion neurons and mouse neuroblastoma cells and they retain many of the characteristics of neurons. They are useful due to the fact that they are well characterized, there are several stresses known which can induce them to undergo apoptotic or non-apoptotic cell death, and additionally they also grow well in culture and when differentiated possess many characteristics of neurons (Wheatley et al., 1992). An additional limitation, as mentioned above, is the fact that the ND7 cell line is not human, whilst the SOD1 cDNAs expressed are human. This could potentially complicate the analysis of data; however, there have been many studies where trans-species experiments have been successful (for examples see Darios et al. (2003) and Katzir et al. (2003)).

Therefore, having established the effects of wt or mutant forms of SOD1 in the neuronal cellular system described above, the following chapter attempts to answer the question of whether mutant SOD1-associated toxicity can be reduced or even abolished by

exogenous over-expression of various Hsps either singly or in combination, with the help of highly efficient HSV-based viral vectors. The experimental evidence provided in the next chapter suggests a novel neuroprotective role for Hsp27 and Hsp70 in combination, in the model of mutant SOD1-associated toxicity, which was described here.

# CHAPTER 4

## The Neuroprotective Effects of Heat Shock Proteins in an *In Vitro* Model of Mutant-SOD1-Induced Toxicity

## 4.1 Introduction

The previous chapter dealt with the establishment and characterisation of an *in vitro* model of mutant SOD1-induced toxicity, where wt-SOD1 has a protective anti-apoptotic effect against cell death induced by a range of death inducing stimuli, whereas overexpression of either G93A or G93R mutant SOD1 enhanced cell death under all stresses tested. Having established these effects, I then proceeded to study the potential neuroprotective effect of various heat shock proteins (Hsps) in this neuronal model system, utilising a highly efficient HSV-based gene delivery system (Wagstaff et al., 1999). This section introduces some of the findings that support a potential protective role of Hsps in neurodegenerative disorders; a more extended review on Hsps is provided in Chapter 1.

Neuroprotection is defined as an intervention that stops, prevents, or slows neuronal degeneration and hence disease progression. Many factors have been involved so far in the aetiology of Amyotrophic Lateral Sclerosis (ALS), providing potential targets for a neuroprotective therapy. Among the possible neuroprotective approaches to list a few, are vitamin E, ascorbate, copper chelators, creatine, and heat shock proteins (see chapter 1 for extended review). The assessment of the protective effect of Hsp27 or of Hsps in combination has not been studied before in the context of mutant SOD1-related toxicity. This chapter provides data that support a novel way by which mutant SOD1-related toxicity and cell death can be alleviated by the overexpression of heat shock proteins, in particular by Hsp27 and Hsp70 in combination (Patel et al., 2005). As a result of these studies, the Hsp27 and Hsp70 combination is suggested to have a novel neuroprotective role that awaits confirmation in *in vivo* systems.

The accumulation of abnormally folded proteins in the nucleus or the cytosol that takes place as a result of stress such as elevated temperature, free oxygen radicals, heavy metals and even antibiotics (Sherman et al., 2001), results in the formation of aggregates that disturb normal cellular function and trigger cell death (Sherman et al., 2001). Such a mechanism has been implicated in the pathogenesis of lesions that are characteristic of a number of neurodegenerative diseases. Neuronal cells seem to be particularly vulnerable to the toxic effects of mutant or misfolded protein aggregates. Most Hsps are involved in the

proper folding and/or elimination of misfolded protein, thereby acting as the first line of defence against it and contributing to cell survival.

SOD1 is a ubiquitously expressed protein and there is no change in its abundance in motor neurons. In SOD1 mutant cells, however, there is a reduction in the activity of molecular chaperones, which results in the accumulation and inefficient removal of the SOD1 mutant (Bruening et al., 1999). The mutated protein may be unstable and as a result more susceptible to precipitation and hence more likely to generate toxic aggregates (Brown. 1995). This is important when taking into consideration that SOD1 represents approximately 1% of cytosolic protein. Changes in folding, solubility, or degradation of such an abundant protein may possibly result in aggregates, consistent with the detection of SOD1 immunoreactive inclusion bodies in motor neurons expressing mutant SOD1 but not with wild-type SOD1 expressed to the same extent (Bredesen et al., 1996; Bruijn et al., 1998; Durham et al., 1997). The formation of SOD1 mutant aggregates is prevented by induction of stress-inducible Hsp70 in cultured neuronal cells, indicating that the decrease in the pool of available chaperones leaves mutant SOD1 cells particularly susceptible to physiological and environmental stress (Bruening et al., 1999).

As yet it remains controversial as to whether cytoplasmic mutant SOD1 aggregates are toxic (Bruijn et al., 1998) or not (Cummings et al., 1999; Johnston et al., 2000; Kopito. 2000). Previous studies in neuronal cells and cultured primary motor neurons have demonstrated that the inhibition of cytoplasmic aggregate formation by induction of heat shock protein Hsp70 assured cell survival at an early stage but did not prevent eventual cell death at the late stage in the *in vitro* models of FALS (Bruening et al., 1999; Takeuchi et al., 2002).

Some of the important house-keeping functions attributed to the molecular chaperones include the import of proteins into cellular compartments; folding of proteins in the cytosol, endoplasmic reticulum, and mitochondria; degradation of unstable proteins; dissolution of protein complexes; prevention of protein aggregation; control of regulatory proteins; as well as refolding of misfolded proteins (Bukau et al., 1998). As reviewed in Chapter 1, it is only in the last few years that reports have started to elucidate the underlying

mechanisms by which Hsps modulate cell death. For instance, Hsp27 expression is seen to correlate with increased survival in response to cytotoxic stimuli and has been shown to prevent cell death by a wide variety of agents that cause apoptosis (Benjamin et al., 1998; Wagstaff et al., 1999). Over the years, evidence has accumulated to show that Hsp27 can inhibit apoptosis by means of a direct inhibition of caspase activation (Garrido et al., 1999; Samali et al., 2001). Hsp70 functions as a major cellular defence molecule against protein aggregation (Glover and Lindquist, 1998) and prevents both caspase-dependent and caspase-independent apoptosis. It is also apparent that such anti-apoptotic functions of Hsps may be distinct from their role as chaperones. In addition a "protein triage" machinery has been proposed in which Hsps and the ubiquitin/proteasome system work together to modulate apoptosis, but further studies are required for a better understanding of the underlying mechanisms. The key inducible Hsps of the nervous system are Hsp27 and Hsp70 and both have been demonstrated to be neuroprotective. In particular, over-expression of Hsp27 in cultured neuronal cells provides protection from induction of apoptosis by a range of stimuli such as neurotrophic factor or serum removal (Wagstaff et al., 1999). Hsp70 enhances the ability of cells to deal with increased concentrations of unfolded or denatured proteins, (Nollen et al., 1999) and is protective against a wide range of lethal stimuli.

Over-expression of Hsp27 protects against apoptotic cell death triggered by various stimuli, including hyperthermia, oxidative stress, staurosporine, ligation of the Fas/Apo-1/CD95 death receptor, and cytotoxic drugs (Garrido et al., 1996 & 1997; Mehlen et al., 1996). Various distinct mechanisms have been put forward to account for the anti-apoptotic properties of Hsp27 such as its ability to increase the anti-oxidant defence of cells by decreasing reactive oxygen species cell content (Mehlen et al., 1996) and its ability to stabilize actin microfilaments (Lavoie et al., 1995). It has been demonstrated that Hsp27 could prevent the formation of the apoptosome and as a result the subsequent activation of caspases (Garrido et al., 1999) by directly sequestering cytochrome *c* when released from the mitochondria into the cytosol (Bruey et al., 2000). At higher intracellular levels, Hsp27 has been shown to hinder caspase activation upstream of the mitochondria (Parcellier et al. 2003). Hsp27 has also been shown to affect the Fas-mediated apoptotic pathway and phosphorylated Hsp27 has been shown to directly interact with Daxx to prevent cell death (Charette et al., 2000).

Like Hsp27, Hsp70 protects cells from a wide variety of lethal stimuli as well as from stress-induced caspase-dependent apoptosis, both upstream and downstream of the death associated mitochondrial events. Initially, Hsp70 was proposed to rescue cells from a later stage of apoptosis than any other known survival enhancing drug or protein. In human cervix carcinoma ME-180 cells, treated with TNFα, Hsp70 over-expression did not prevent activation of caspase-3 but inhibited downstream morphological alterations that are characteristically associated with dying cells (Jaattela et al., 1998). It was subsequently found that elevated levels of Hsp70 could prevent both caspase activation and nuclear changes associated with apoptosis (Buzzard et al., 1998). In the intrinsic pathway to cell death, over-expression of Hsp70 did not inhibit mitochondrial cytochrome c release; however, caspase-3 activation was prevented (Li et al., 2000). These observations were subsequently linked to the ability of Hsp70 to directly bind Apaf-1 through its ATPase domain, and consequently prevent the recruitment of procaspase-9 to the apoptosome (Saleh et al., 2000; Mosser et al., 2000; Beere et al., 2000).

Although studies have examined the protective effects of over-expression of Hsps, none have investigated comparatively the protective effects of Hsp27 or Hsp70 over-expression, or combined effects of both together, with respect to FALS-associated SOD1 mutants. In the present project, herpes simplex viruses were utilised in order to assess whether Hsp27, Hsp70 or the combination of Hsp27 and Hsp70 have any protective effect against various stresses applied in an *in vitro* system in which wt-SOD1 or the disease-associated mutants, G93A and G93R-mutant SOD1, are stably over-expressed. Cells were subjected to a wide range of stresses such as serum removal, serum removal plus 1μM *all-trans* retinoic acid, IFN-γ treatment, staurosporine administration and camptothecin addition, in order to compare their response to these distinct death stimuli, in the presence or absence of over-expressed Hsps, at multiple time points.

These stresses predominantly result in apoptotic cell death, which was assessed by the trypan blue exclusion assay and TUNEL staining. The trypan blue exclusion assay is an easy and relatively simple yet reliable and inexpensive method of assessing total cell death and it is based on the fact that cell membrane permeability to trypan blue dye depends on membrane integrity; with non-viable cells lacking the ability to exclude the dye and hence

staining blue. TUNEL analysis is a frequently used technique for the detection of apoptosis that utilises TdT-mediated X-dUTP nick end labelling (where X is a suitable conjugated label such as rhodamine or FITC), however its specificity is not absolute as DNA fragmentation is also found to be common in other types of cell death.

In order to test the potential neuroprotective role of various Hsps in this model system, it was important to utilize a highly efficient Hsp gene delivery system. Therefore in this present study HSV-based viral vectors were chosen. These viral vectors have been developed specifically for the efficient delivery of the gene of interest to neuronal cells and their use has been reported in both *in vitro* and *in vivo* studies (Wagstaff et al., 1999; Brar et al., 1999; Kalwy et al., 2003). The suitability and effectiveness of HSV-based gene delivery, was discussed in Chapter 1 (for review see Latchman and Coffin, 2001; Davidson et al., 2003 and Goins et al., 2004).

Briefly, the viruses used in this present study, provide a highly efficient method of specific gene delivery to ND7 cells as well as primary neuronal cells (90-100% transduction efficiency). The viral vector used in this study was constructed in our laboratory by Dr Marcus Wagstaff and named 17+pR19 (Wagstaff et al., 1998). The transgenes (Chinese hamster hsp27, human inducible hsp70, or GFP) in this virus are driven by the human CMV-IE promoter, inserted immediately downstream of the LAT P2 promoter. Figure 4.1 below shows the expression cassette, which was inserted into the non-essential 2 kb LAT region of the HSV-1 genome of an IE2-deleted mutant HSV-1 strain (IE2 is the essential gene that encodes ICP27; HSV-1 strain 17+; GenBank™ accession number HE1CG) (Wagstaff et al., 1999). The IE genes ICP4 and ICP27 are essential for viral replication. Therefore ICP27 was deleted in this virus type. These viruses were designed to produce a less cytopathic, latent, non-replicating viral mutant, which was tested and found to be suitable for gene delivery (Wagstaff et al., 1999; Kalwy et al., 2003). These viruses were grown on B130/2 cells which complement for the deleted ICP27 (Howard et al., 1998).

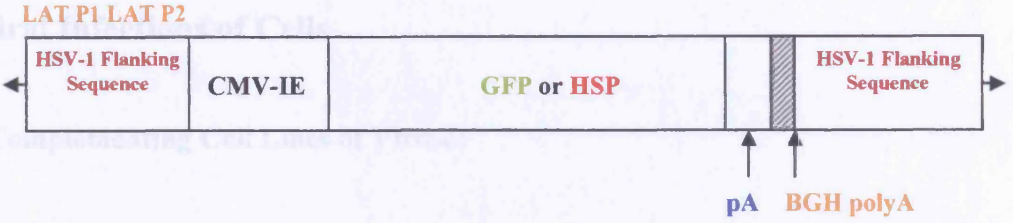



**Figure 4.1** The pR19 cassette utilised in the 17+ virus constructs.

This expression construct was introduced into the non-essential LAT region of the IE2 deleted mutant HSV-1 DNA. The CMV-IE promoter drives the transcription of the transgene as well as the bovine growth hormone polyadenylation sequence. The use of the LAT P1 and LAT P2 promoters with the CMV-IE promoter is thought to facilitate expression throughout viral latency in neurons.

In this present study the availability of a highly efficient HSV-1-based vector system capable of delivering Hsp genes to neuronal cells, and the availability of characterised stable cell lines expressing wt-SOD1 and its mutant forms proved to be very useful. As shown in this chapter, it allowed us to test the hypothesis of whether mutant-SOD1-related toxicity in mammalian neuronal cells subjected to various stresses can be suppressed by individual or by combinations of heat shock proteins (Hsp27, Hsp70).

The following sections of this chapter briefly describe the method of viral infection of the cell lines, the characterisation of heat shock protein expression in the cell lines, followed by a description of the results obtained on exposure to each death stimulus along with quantification of cell death. Finally, the significance of the findings is discussed, along with the advantages and limitations of the model system.

## 4.2 Viral Infections of Cells

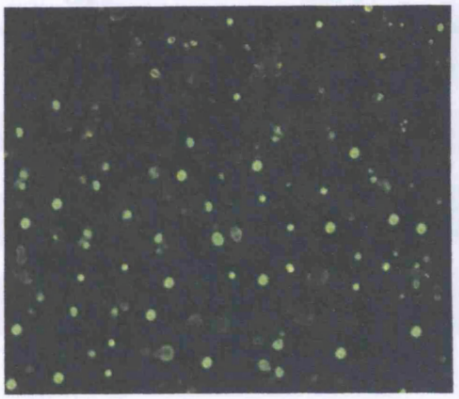
### 4.2.1 Complementing Cell Lines of Viruses

The stocks of virus used in this study were grown and prepared as described in Chapter 2, section 2.4. The viruses were grown on B130/2 BHK cells (used for 17+27- virus type) or M49 cells (used for 1764 27-4- virus type) as they complement the deletions in the disabled viruses and hence enable lytic viral growth (Howard et al., 1998; Thomas et al., 1999). This allows the production of large quantities of the virus, which can be subsequently purified and concentrated into high titre stocks. Each preparation of high titre stock was confirmed to express the transgene, through western analysis of non-complementing ND7 cells infected with the stock.

### 4.2.2 Microscopy of Virally Infected ND7 Cells and Western Blot Analysis

ND7 cells were infected as described in Chapter 2, section 2.4.2 and then left for 24h to allow overexpression of the transgene prior to treatment. However, GFP was visible earlier than 12h post-infection, and its expression continued to be seen until the end of all treatments. Figure 4.2a shows some ND7 cells infected with GFP virus. The HSV-vector 17+pR19 has previously been shown to successfully infect ND7 cells and produce large amounts of Hsps (Wagstaff et al., 1999). Figure 4.3 shows the high levels of expression of the transgene after 24h in ND7 cells infected with either a control GFP, Hsp27 or Hsp70 expressing virus. The GFP virus enables a relatively quick assessment of the percentage of cells expressing GFP and is used as a method of assessing efficiency of infection of the Hsp viruses, as the Hsp expressing viruses do not contain a visual marker; the assumption made is cells infected with the same amount of virus and at the same m.o.i, will result in the same transduction efficiency.

(a)



(b)

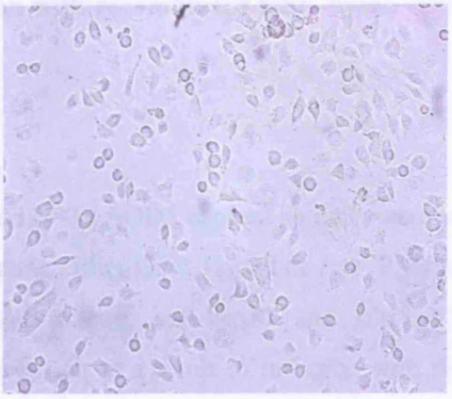


**Figure 4.2 Gene delivery of GFP or heat shock proteins using HSV-based viral vectors.**

**(a)** ND7 cells 24h following infection with GFP virus and **(b)** ND7 cells 24h following infection with Hsp27 virus at 10 m.o.i. Cells exhibit normal morphology. The Hsp viruses do not possess a visual marker in order to enable overexpression of the appropriate Hsp to be observed, therefore the expression levels were assessed by means of Western blotting (Figures 4.3).

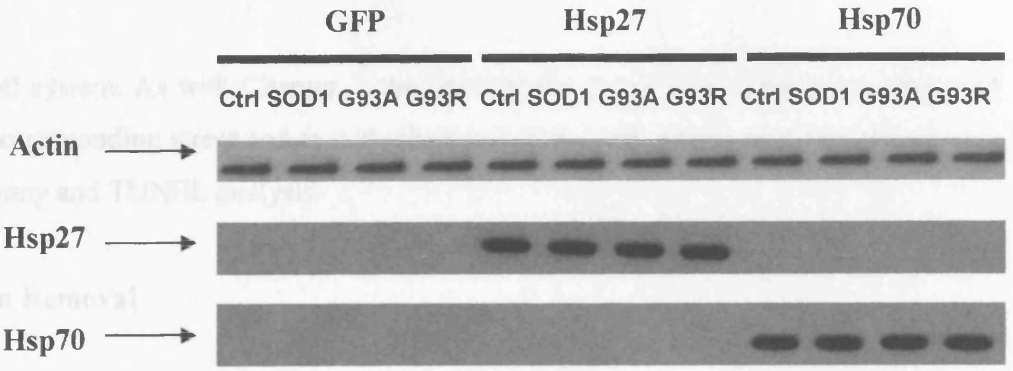



**Figure 4.3. Heat shock protein over expression in ND7 cells expressing wild type or mutant SOD1 at 24h following infection with recombinant HSV-based vectors expressing GFP, Hsp27 or Hsp70.**

Control, wt and mutant G93A and G93R SOD1 expressing cells were infected at an m.o.i of 10 with recombinant viruses over-expressing either GFP, Hsp27 or Hsp70; after which western blotting was carried out with antibodies: anti-β-actin, anti-hsp27 and anti-hsp70 antibodies. There is no significant induction of Hsp expression as a result of infection with the virus itself and this can be seen with the GFP control virus, however, there is high expression of the transgenes, all driven by the CMV-IE promoter.

## 4.3 Responses of the SOD1 cell lines to Stress following Exogenous Heat Shock Protein Over-expression

Having characterised the expression of wt and mutant SOD1 in the ND7 cells (see Chapter 3) and having demonstrated that Hsps can be over expressed in those cells through use of viral vectors, the next step was to utilise this system and test whether or not Hsps can confer neuroprotection against the toxic effects of the FALS-associated mutant forms of SOD1 *in vitro*. This section therefore describes the results obtained following experimentation into the protective effect of individual Hsps as well as Hsps in combination, in this model system. As with Chapter 3, the findings for the various stresses are presented

in this model system. As with Chapter 3, the findings for the various stresses are presented below the corresponding stress and as with chapter 3, cell death was assessed by trypan blue exclusion assay and TUNEL analysis.

### 4.3.1 Serum Removal

Therefore, conditions of serum removal (both in the absence and presence of 1μM retinoic acid; for constituents of serum free media see Methods and Materials) were applied to the various cell lines for periods of 24h and 48h. Hsp27 protected both G93A and G93R mutant-SOD1 over-expressing cells from serum deprivation with death being reduced by approximately 12% for G93A and 31% for G93R, compared to the respective GFP controls, at 24h time point ($p<0.0005$) (Figure 4.4a). In addition Hsp70 virus also conferred protection with death being reduced by approximately 9% for G93A and 41% for G93R, compared to the respective GFP controls, at 24h time point ($p<0.01$ and $p<0.0005$ respectively). However the wt-SOD1 over-expressing cells which are already protected under this stress, when infected with Hsp27 showed an increase in death with death rising by approximately 14%, compared to the wt-SOD GFP control, at 24h time point ($p<0.01$). A more dramatic increase was observed when the wt-SOD1 cells were infected with Hsp70 with death rising by nearly 93%, compared to the wt-SOD GFP control, at 24h time point ($p<0.0005$); (Figure 4.4a).

When a joint viral infection of Hsp27 and Hsp70 (Total m.o.i used = 10; m.o.i Hsp27=5, m.o.i Hsp70=5) was undertaken the results showed a greater reduction in death than was obtained by the respective single infections with death decreasing by almost 22% and 44% for G93A and G93R mutant respectively, compared to respective GFP controls, at 24h ($p<0.0005$); (Figure 4.4a). With respect to wt-SOD1 the joint infection brought cell death down to control GFP levels although this was still higher than that of uninfected wt-SOD1 control. Similar results were obtained at the 48h time point, with the protective effect of the Hsps still able to be seen very clearly in the mutant SOD over-expressing cells with death decreasing by 32% and 47% for G93A and G93R mutant respectively, compared to respective GFP controls ($p<0.0005$); (Figure 4.4a). TUNEL analysis gave similar results strengthening the evidence for an anti-apoptotic/protective role of Hsp27 and Hsp70. Hsp27

and Hsp70 both reduced apoptosis significantly compared to GFP in cells expressing both G93A and G93R mutant SOD1 (Figure 4.4b). A dual Hsp infection of both G93A and G93R mutant SOD1 over-expressing cells significantly lowered apoptosis, even further than either Hsp27 or Hsp70 virus singly ($p<0.0005$) (Figure 4.4b).

Figure 4.4a

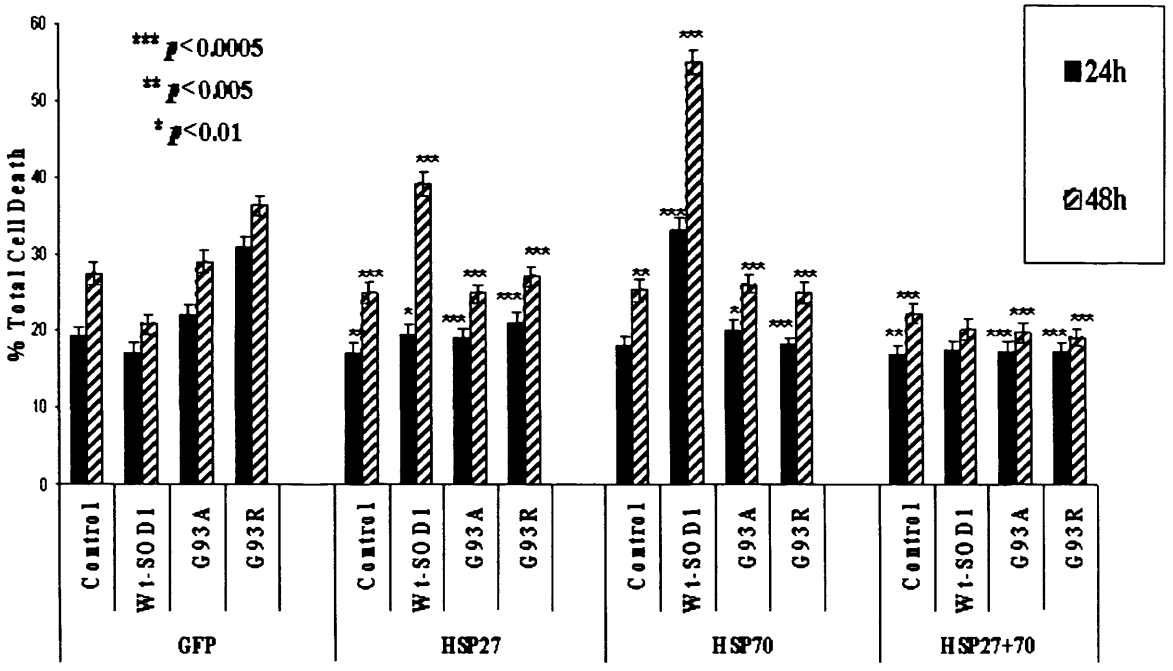



**Figure 4.4 Effect of serum withdrawal in SOD1 stable cells following Hsp infection**

**a.** ND7 cell death following 24h and 48h serum removal after infection with HSV vectors expressing Hsps. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h and 48h of serum deprivation of engineered ND7 cells expressing wt or mutant SOD1. Cells were infected with Hsp viruses 16h prior to stress. Bars represent mean $^{+}$S.D. calculated for triplicate counts per sample (n), n=5. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p<0.0005$). *, **, *** indicates statistically significant difference between means, when comparing with the respective GFP virus infected cell line.

Figure 4.4b

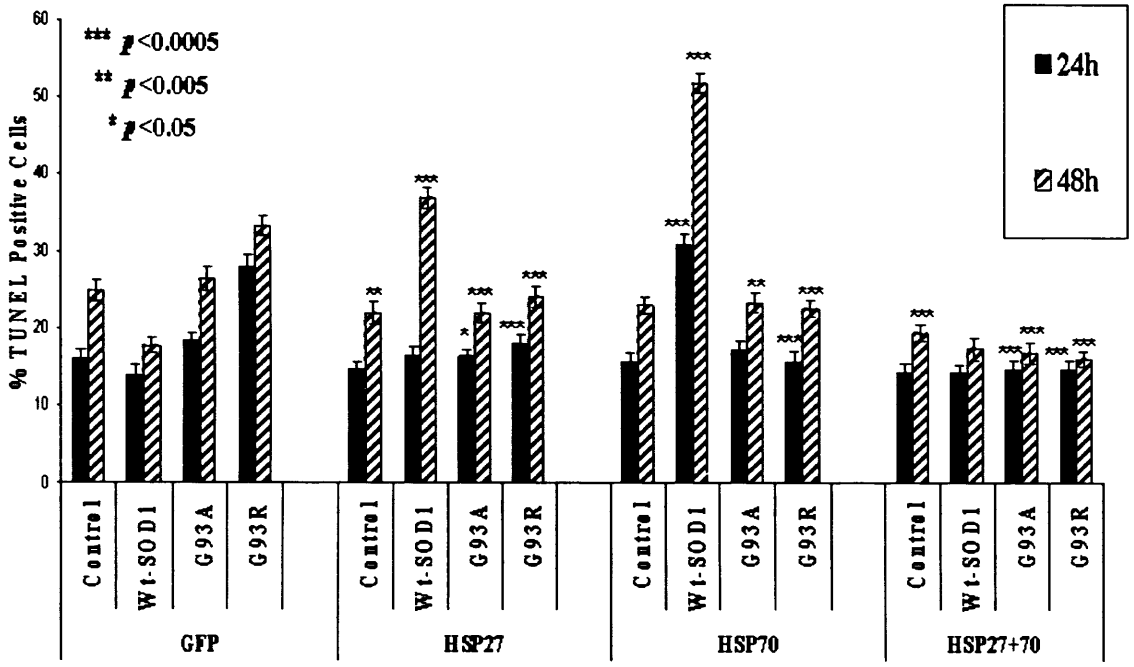



**Figure 4.4 Effect of serum withdrawal in SOD1 stable cells following Hsp infection**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were infected with viruses to over-express Hsps or GFP 16h prior to treatment of serum deprivation for 24h and 48h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=3) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with the respective GFP virus infected cell line.

### 4.3.2 Serum Removal+1μM Retinoic Acid

Having established that both Hsp27 and Hsp70 had a protective effect against serum deprivation on both G93A and G93R SOD1 mutants and a damaging effect on wt-SOD1 over-expressing cells, we further investigated the effect of the Hsp viruses on the SOD1 mutants and wt-SOD1 over-expressing cells when subjected to other death-inducing stimuli. Serum withdrawal in the presence of all-trans-retinoic acid stimulates higher levels of apoptosis (Howard et al., 1993; Wagstaff et al., 1999).

The delivery of Hsps via the HSV viral vector system showed both Hsp27 and Hsp70 having a protective effect on both mutants with death being reduced for both SOD1 mutants compared to their respective GFP infected controls ($p<0.0005$) (Figure 4.5a). Wt-SOD1 over-expressing cells exhibited higher levels of death on infection with Hsp27 and also on infection with Hsp70 with the increase in death being statistically significant compared to GFP infected control ($p<0.0005$) (Figure 4.5a). On infection with Hsp27, the level of death in wt-SOD1 cells increased by approximately 34% and 80% at 24h and 48h respectively compared to GFP control ($p<0.0005$). While on infection with Hsp70, the level of death in wt-SOD1 cells increased by approximately 73% and 144% at 24h and 48h respectively compared to GFP control ($p<0.0005$). Hsp27 infection of the G93A mutant cells reduced death by 38% and 32% at the 24h and 48h time points compared to the respective GFP control ($p<0.0005$). Hsp27 infection of G93R mutant cells brought about an approximately 49% and 46% reduction in total cell death at 24h and 48h respectively, compared to GFP infected control ($p<0.0005$). Hsp70 infection of mutants reduced cell death by approximately 36% and 18% for G93A and 53% for G93R at 24h and 48h respectively compared to respective GFP control ($p<0.0005$).

A combinational treatment of dual Hsp27 and Hsp70 gave a greater reduction in death for both mutants with death being reduced by approximately 50% and 48% for G93A and 60% and 59% for G93R, at the 24h and 48h time points, with the reduction in death being statistically significant compared to the respective GFP infected controls at these time points ($p<0.0005$) (Figure 4.5a). The joint infection of Hsp27 and Hsp70 on the wt-SOD1 over-

expressing cells also brought the exacerbated death caused by the single infections Hsp27 and Hsp70 down to control GFP levels.

Figure 4.5b shows the number of TUNEL positive cells is significantly reduced on infection with Hsp27 and also Hsp70 in control and both G93A and G93R SOD1 mutants ($p<0.0005$). Figure 4.5b also shows the significant and greater reduction in cell death of both G93A and G93R SOD1 mutants that follows dual Hsp27 and Hsp70 infection ($p<0.0005$). On infection with Hsp27 the number of TUNEL positive cells were reduced in the G93A mutant by 48% and 40% at the 24h and 48h time points compared to GFP control ($p<0.0005$). In the case of G93R mutant death was reduced by 61% and 55% at 24h and 48h compared to GFP infected control ($p<0.0005$). Hsp70 infection of G93A reduced death by 47% and 24% at 24h and 48h respectively compared to GFP control ($p<0.0005$). In the case of the G93R mutant, Hsp70 infection reduced death by approximately 64% at both 24h and 48h time points compared to GFP infected control ($p<0.0005$). The greatest protection was seen on dual infection of both Hsp27 and Hsp70. Dual infection in the G93A mutant reduced death by approximately 61% and 63% at 24h and 48h time points respectively compared to GFP control ($p<0.0005$). In the G93R mutant death was reduced by approximately 70% at both 24h and 48h time points on dual infection of Hsp27 and Hsp70 when compared to respective GFP control ($p<0.0005$). Infection of wt-SOD1 cells with Hsp27 led to an increase in cell death with death being increased by 52% and 115% at 24h and 48h time points respectively compared to respective GFP infected control ($p<0.0005$). Hsp70 infection of wt-SOD1 cells increased death by an even greater extent with death being increased by 106% and 206% at the 24h and 48h time points respectively compared to respective GFP control ($p<0.0005$). On dual infection of wt-SOD1 cells with Hsp27 and Hsp70 the death was reduced back down to control GFP levels at both 24h and 48h time points.

Figure 4.5a

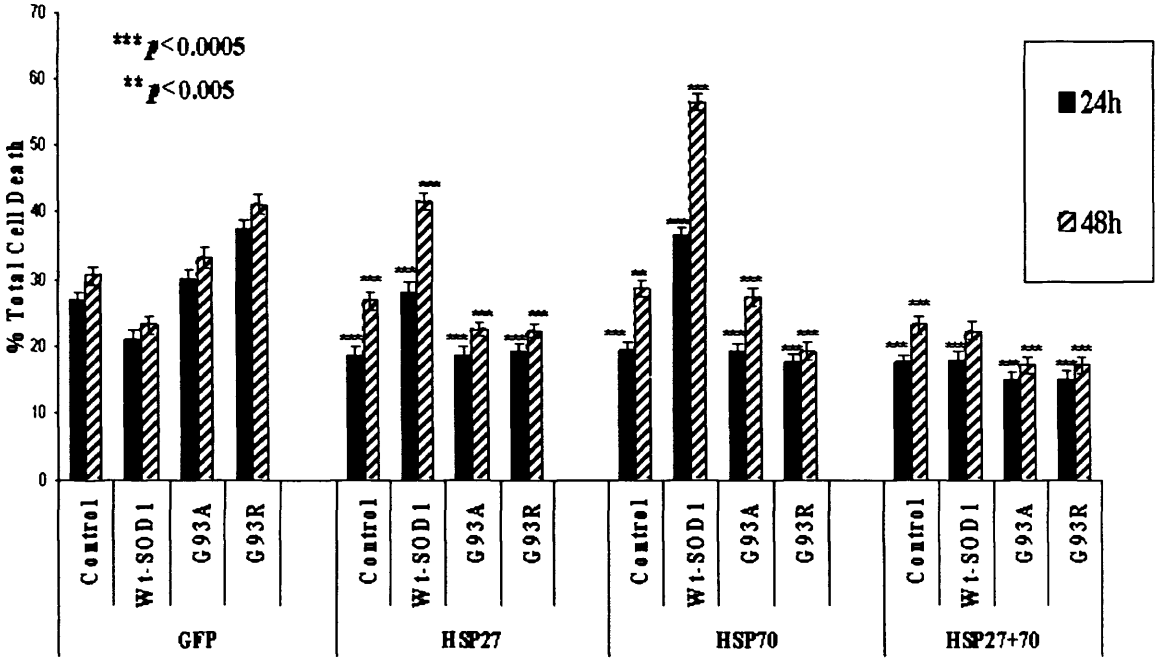



**Figure 4.5 Effect of serum withdrawal plus 1μM *all-trans* retinoic acid in SOD1 stable cells following Hsp infection**

**a.** ND7 cell death following 24h and 48h serum removal plus 1μM *all-trans* retinoic acid after infection with HSV vectors expressing Hsps. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h and 48h of serum deprivation plus 1μM *all-trans* retinoic acid of engineered ND7 cells expressing wt or mutant SOD1. Cells were infected with Hsp viruses 16h prior to stress. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=5. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p$<0.0005). *, **, *** indicates statistically significant difference between means, when comparing with the respective GFP virus infected cell line.

Figure 4.5b

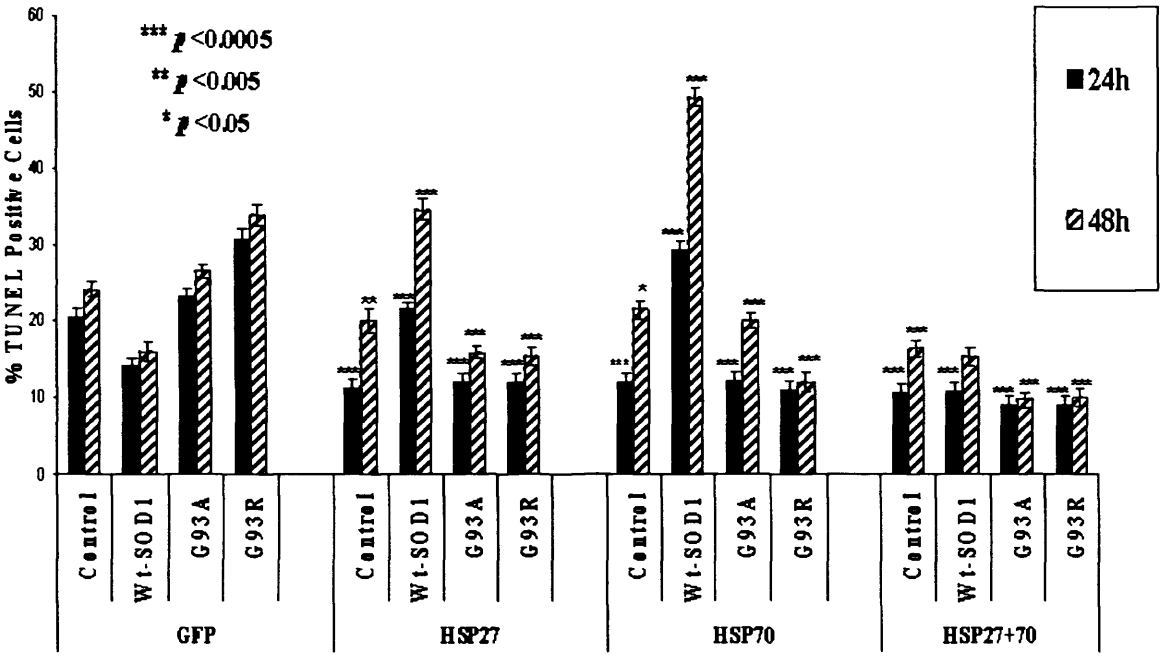



**Figure 4.5 Effect of serum withdrawal plus 1μM *all-trans* retinoic acid in SOD1 stable cells following Hsp infection**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were infected with viruses to over-express Hsps or GFP 16h prior to treatment of serum deprivation plus *all-trans* retinoic acid for 24h and 48h. Bars represent mean $^{\pm}$ S.D. calculated for triplicate counts per sample (n=3) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with the respective GFP virus infected cell line.

### 4.3.3 IFN-γ administration

Subsequently, the cells were treated with IFN-γ for 24h and 48h which induces cell death in a variety of neuronal and non-neuronal cell types (Figure 4.6a). A clear marked protective effect of Hsp27 and Hsp70 was observed compared to control GFP, in cells expressing both G93A and G93R SOD1 mutants ($p<0.0005$) (Figure 4.6a). As with serum removal and serum removal plus all-trans retinoic acid experiments, Hsp27 greatly reduced cell death for both mutants as did Hsp70. Death was reduced by approximately 30% and 32% for G93A and 41% and 44% for G93R following Hsp27 infection at 24h and 48h time points respectively compared to respective GFP controls. Hsp70 infection lowered death by approximately 23% and 27% for G93A and 45% and 53% for G93R respectively at 24h and 48h, compared to respective GFP control ($p<0.0005$); (Figure 4.6a). Again the dual viral infection of Hsp27 and Hsp70 together gave a further reduction in death in both mutants with death being reduced by 33% and 40% for G93A and 52% and 56% for G93R respectively, at 24h and 48h respectively, compared to the respective GFP controls ($p<0.0005$); (Figure 4.6a). The wt-SOD1 over-expressing cells showed increased levels of death on infection with Hsp27 and further increased levels on infection with Hsp70, with a dual infection again bringing levels down to that of wt-SOD1 GFP infected control. Death increased in the wt-SOD1 cells by 41% and 49% at 24h and 48h, compared to respective GFP control, on infection with Hsp27 ($p<0.0005$). Hsp70 infection increased death in the wt-SOD1 cells by an even greater extent with death rising by approximately 101% and 128% at 24h and 48h respectively, compared to respective GFP control ($p<0.0005$). Dual infection of Hsp27 and Hsp70 in the wt-SOD1 cells reduced death back to control GFP levels.

Similarly, as assessed by TUNEL, Hsp27, Hsp70 and the combination of Hsp27 & Hsp70 significantly reduced the number of TUNEL positive cells in both G93A and G93R SOD1 mutants ($p<0.0005$), compared to GFP/G93A or GFP/G93R over-expressing cells (figure 4.6b). Infection of G93A cells with Hsp27 resulted in a reduction in the number of TUNEL positive cells by approximately 33% and 42% at 24h and 48h respectively compared to control ($p<0.0005$). Hsp70 infection of G93A was not as effective as Hsp27 in reducing the number of TUNEL positive cells with a reduction of approximately 25% and 34% being

observed at 24h and 48h respectively compared to control ($p<0.0005$). In the G93R cells, Hsp27 infection reduced the number of TUNEL positive cells by approximately 49% and 48% at 24h and 48h respectively compared to control ($p<0.0005$). Hsp70 infection of the G93R cells was much more effective in reducing the number of TUNEL positive cells than Hsp27 with the numbers being reduced by approximately 52% and 57% at 24h and 48h respectively compared to control ($p<0.0005$). The dual infection of Hsp27 and Hsp70 was the most effective in reducing the number of TUNEL positive cells with the number being reduced by approximately 41% and 47% for G93A and 60% and 62% for G93R at 24h and 48h respectively compared to control ($p<0.0005$). Wt-SOD1 cells exhibited an increase in the number of TUNEL positive cells on infection with Hsp27 or Hsp70 with death increasing by a approximately 46% and 64% on Hsp27 infection and more dramatically by 129% and 158% on Hsp70 infection at 24h and 48h respectively compared to control ($p<0.0005$). The dual infection of Hsp27 and Hsp70 reduced the number of TUNEL positive cells back down toward control GFP levels in the wt-SOD1 cells.

Figure 4.6a

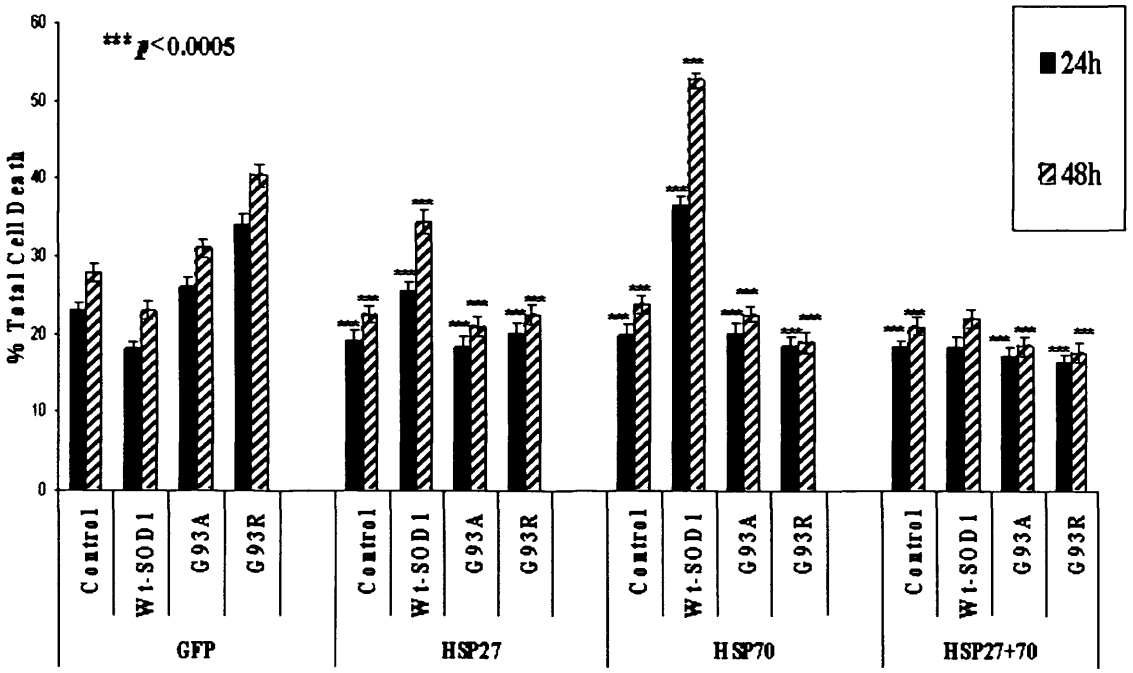



**Figure 4.6 Effect of IFN-γ in SOD1 stable cells following Hsp infection**

**a.** ND7 cell death following 24h and 48h treatment with 50ng/ml IFN-γ after infection with HSV vectors expressing Hsps. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h and 48h treatment with IFN-γ of engineered ND7 cells expressing wt or mutant SOD1. Cells were infected with Hsp viruses 16h prior to stress. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=5. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p<0.0005$). *, **, *** indicates statistically significant difference between means, when comparing with the respective GFP virus infected cell line.

Figure 4.6b

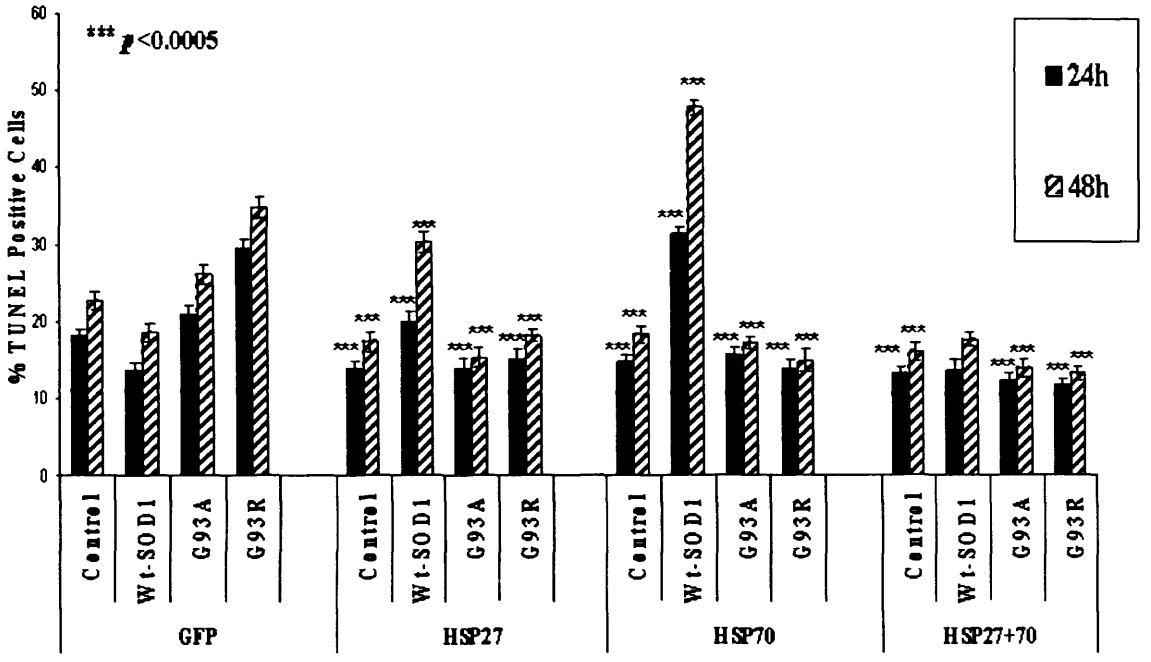



**Figure 4.6 Effect of IFN-γ in SOD1 stable cells following Hsp infection**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were infected with viruses to over-express Hsps or GFP 16h prior to treatment of 50ng/ml IFN-γ for 24h and 48h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=3) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with the respective GFP virus infected cell line.

### 4.3.4 Staurosporine

A time course experiment was subsequently carried out to analyse the response of the cells to treatment with staurosporine (Figure 4.7a). A 1μM concentration of staurosporine increased the total cell death from 27% and 30% at 2h to 43% and 46% at 6h for G93A and G93R mutants respectively (Figure 4.7a). On infection with the Hsp27 and Hsp70 viruses the death in both mutants was again significantly reduced. In the G93A mutant Hsp27 infection reduced death by approximately 35%, 29% and 22% at 2h, 4h and 6h respectively compared to respective GFP control ($p<0.0005$). Hsp27 infection of the G93R mutant cells reduced death by approximately 43%, 32% and 39% at 2h, 4h and 6h respectively, compared to respective GFP control ($p<0.0005$). Hsp70 infection of G93A mutant cells lowered death by 20%, 25% and 18% at 2h, 4h and 6h respectively compared to respective GFP control ($p<0.0005$). Infection of G93R mutant with Hsp70 led to a greater reduction in death than that seen with Hsp27 with death being reduced by approximately 48%, 40% and 44% at 2h, 4h and 6h respectively compared to respective GFP control ($p<0.0005$).

The combination of Hsp27 and Hsp70 again appeared to be even more effective in attenuating cell death (Figure 4.7a) in both G93A and G93R SOD1 mutants with cell death being reduced by 45%, 36% and 38% at 2h, 4h and 6h respectively in the case of G93A compared to respective GFP control and for G93R mutant cells 57%, 48% and 46% at 2h, 4h and 6h respectively compared to respective GFP control ($p<0.0005$). Again as with the insults described above, both Hsp27 and Hsp70 individually enhanced cell death in wt-SOD1 over-expressing cell line with the combination reducing death back down to control levels. Infection of wt-SOD1 cells with Hsp27 increased death by approximately 46%, 56% and 42% at 2h, 4h, and 6h respectively compared to respective GFP control ($p<0.0005$). Hsp70 infection of wt-SOD1 cells increased death by a greater amount than Hsp27 with death being increased by approximately 106%, 68% and 54% at 2h, 4h and 6h respectively compared to respective GFP control ($p<0.0005$). Dual infection of Hsp27 and Hsp70 brought the levels of death in the wt-SOD1 cells back down to control GFP levels.

Assessment of death by TUNEL showed Hsp27, Hsp70 and the combination of Hsp27 & Hsp70 significantly reduced the number of TUNEL positive cells in both G93A and G93R

SOD1 mutants ($p<0.0005$), compared to respective GFP infected control cells (figure 4.7b). On Hsp27 infection G93A cells exhibited a reduction in the number of TUNEL positive cells by approximately 34%, 31%, and 24% at 2h, 4h and 6h respectively compared to control ($p<0.0005$). Hsp70 infection of G93A was not as effective in reducing the number of TUNEL positive cells as Hsp27 but effective nonetheless with numbers reduced by approximately 22% ($p<0.005$), 27% ($p<0.0005$) and 21% ($p<0.0005$) at 2h, 4h, and 6h respectively compared to control. The combination of Hsp27 and Hsp70 was the most effective with the death being reduced by approximately 49%, 37% and 40% at 2h, 4h and 6h respectively compared to control ($p<0.0005$). In the G93R cells, Hsp27 infection reduced the number of TUNEL positive cells by approximately 52%, 33% and 42% at 2h, 4h and 6h respectively compared to control ($p<0.0005$). Hsp70 infection was more effective than Hsp27 in the G93R cells with number of TUNEL positive cells being reduced by approximately 53%, 38% and 44% at 2h, 4h and 6h respectively compared to control ($p<0.0005$).

The dual combination of Hsp27 and Hsp70 reduced the numbers of TUNEL positive cells by approximately 60%, 51% and 49% at 2h, 4h and 6h respectively compared to control ($p<0.0005$) and once again was more effective than single infections. Infection of wt-SOD1 cells with Hsp27 resulted in an increase in the numbers of TUNEL positive cells with numbers increasing by approximately 41%, 63% and 52% at 2h, 4h and 6h respectively compared to control ($p<0.0005$). Hsp70 infection of wt-SOD1 cells resulted in more dramatic increase in the number of TUNEL positive cells with numbers increasing by approximately 123%, 80% and 67% at 2h, 4h, and 6h respectively compared to control ($p<0.0005$). Once again as seen with other stresses the dual Hsp27 and Hsp70 infection reduced the increases seen on single infections back down to control levels with numbers reduced by approximately 16% ($p<0.05$), 19% ($p<0.0005$) and 18% ($p<0.0005$) at 2h, 4h, and 6h respectively compared to control.

Figure 4.7a

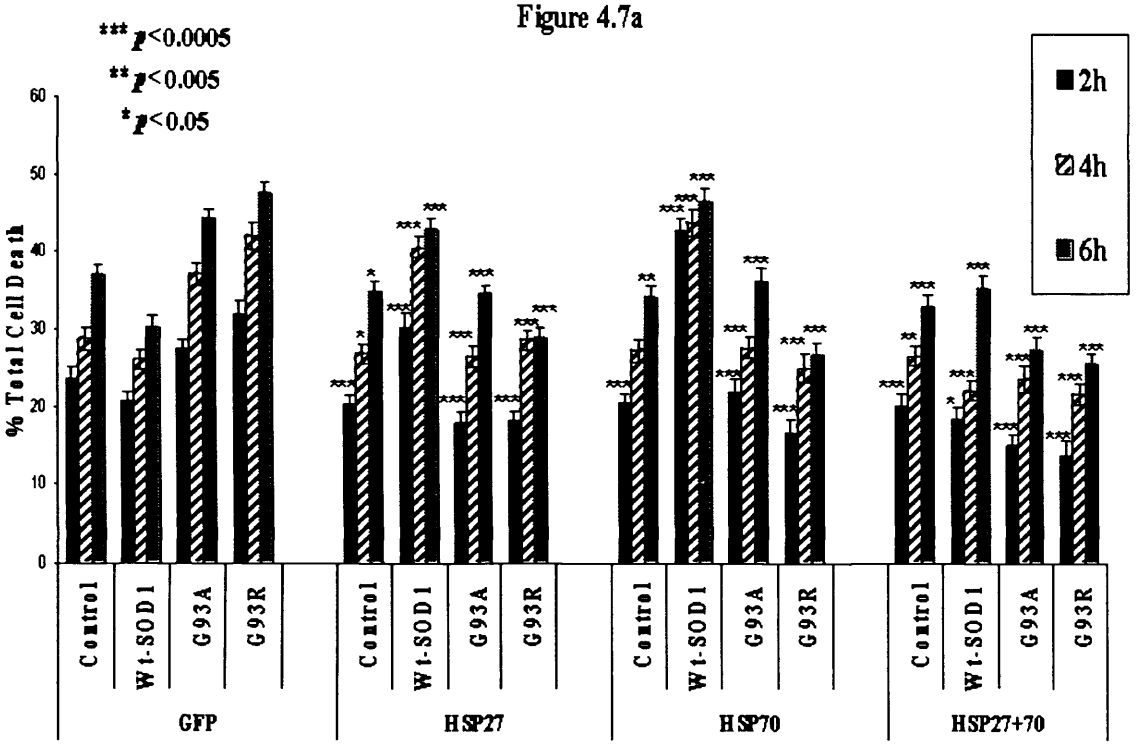



**Figure 4.7 Effect of staurosporine in SOD1 stable cells following Hsp infection**

**a.** ND7 cell death following 2h, 4h and 6h of 1μM staurosporine administration after infection with HSV vectors expressing Hsps. The proportion of cell death was assessed by trypan blue exclusion assay, after 2h, 4h and 6h 1μM staurosporine administration of engineered ND7 cells expressing wt or mutant SOD1. Cells were infected with Hsp viruses 16h prior to stress. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=5. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p<0.0005$). *, **, *** indicates statistically significant difference between means, when comparing with the respective GFP virus infected cell line.

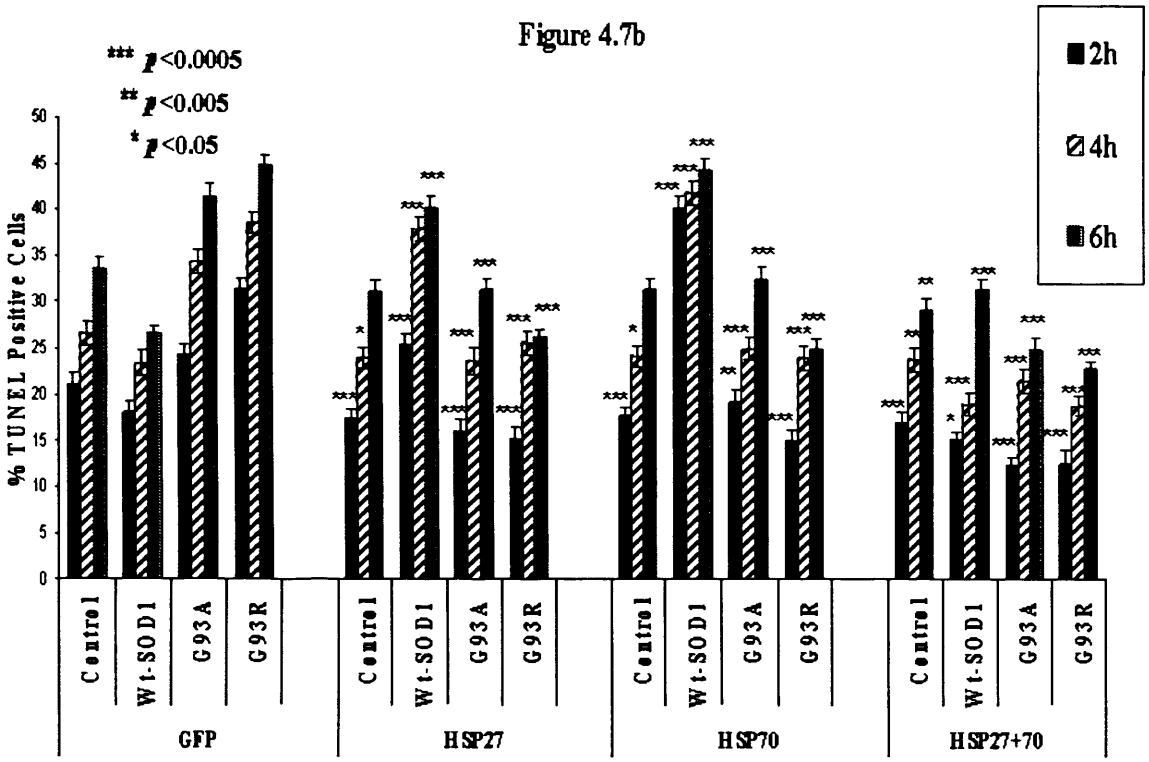



**Figure 4.7 Effect of staurosporine in SOD1 stable cells following Hsp infection**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were infected with viruses to over-express Hsps or GFP 16h prior to 1μM staurosporine administration for 2h, 4h and 6h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=3) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with the respective GFP virus infected cell line.

### 4.3.5 Camptothecin administration

Similar results were also observed when the cells were treated with camptothecin (Figure 4.8a). As with the previous insults, a statistically significant reduction in cell death was observed in the G93A and G93R SOD1 over-expressing mutants following Hsp27 and Hsp70 viral infection compared to respective GFP infected control ($p<0.0005$). Cell death was reduced by 27% and 24% for G93A and 41% and 27% for G93R at 24h and 48h respectively following Hsp27 infection, compared to respective GFP control ($p<0.0005$). Infection with Hsp70 reduced death by approximately 18% and 19% for G93A and 46% and 34% for G93R at 24h and 48h respectively compared to respective GFP control ($p<0.0005$). Wt-SOD1 cells exhibited enhanced cell death with cell death increasing by 61% and 88% at 24h and 48h respectively following Hsp27 infection and 145% and 160% at 24h and 48h respectively following Hsp70 infection, compared to respective GFP control ($p<0.0005$); (Figure 4.8a). As with the previous experiments, the greatest amount of protection was observed when the Hsp27 and Hsp70 viruses were used in combination with protection seen across all cell lines with even wt-SOD1 over-expressing cells showing a reduction in death, down to control GFP levels. On dual infection, death was reduced by 35% and 38% for G93A at 24h and 48h respectively compared to respective GFP control and 49% and 46% at 24h and 48h respectively for G93R compared to respective GFP control ($p<0.0005$).

TUNEL assessment of death again showed Hsp27, Hsp70 and the combination of Hsp27 & Hsp70 significantly reduced the number of TUNEL positive cells in both G93A and G93R SOD1 mutants ($p<0.0005$), compared to GFP/G93A or GFP/G93R over-expressing cells at 24h (figure 4.8b). Hsp27 infection of G93A and G93R cells reduced the number of TUNEL positive cells by approximately 32% at both 24h ($p<0.0005$) and 48h ($p<0.005$) for G93A and 52% and 29% at 24h and 48h for G93R cells compared to control ($p<0.0005$). Hsp70 infection of G93A cells was slightly less effective than Hsp27 in this case with the number of TUNEL positive cells reduced by approximately 21% ($p<0.0005$) and 12% ($p<0.005$) at 24h and 48h respectively compared to control. However in the G93R cells Hsp70 infection was slightly more effective in reducing the number of TUNEL positive cells than Hsp27 with numbers reduced by approximately 55% and 36% at 24h and 48h respectively compared to control ($p<0.0005$)). Hsp27 infection of wt-SOD1 brought about an increase in

the number of TUNEL positive cells with numbers increasing by approximately 89% and 109% at 24h and 48h respectively compared to control ($p<0.0005$). Hsp70 infection of wt-SOD1 cells brought about a more dramatic increase with numbers increasing by approximately 194% and 201% at 24h and 48h respectively ($p<0.0005$)). The dual Hsp27 and Hsp70 infection reduced levels of TUNEL positive cells toward those seen in control. However at 24h the number of TUNEL positive cells was still up by approximately 21% compared to control ($p<0.05$) and at 48h up by approximately 8% compared to control ($p<0.05$).

Figure 4.8a

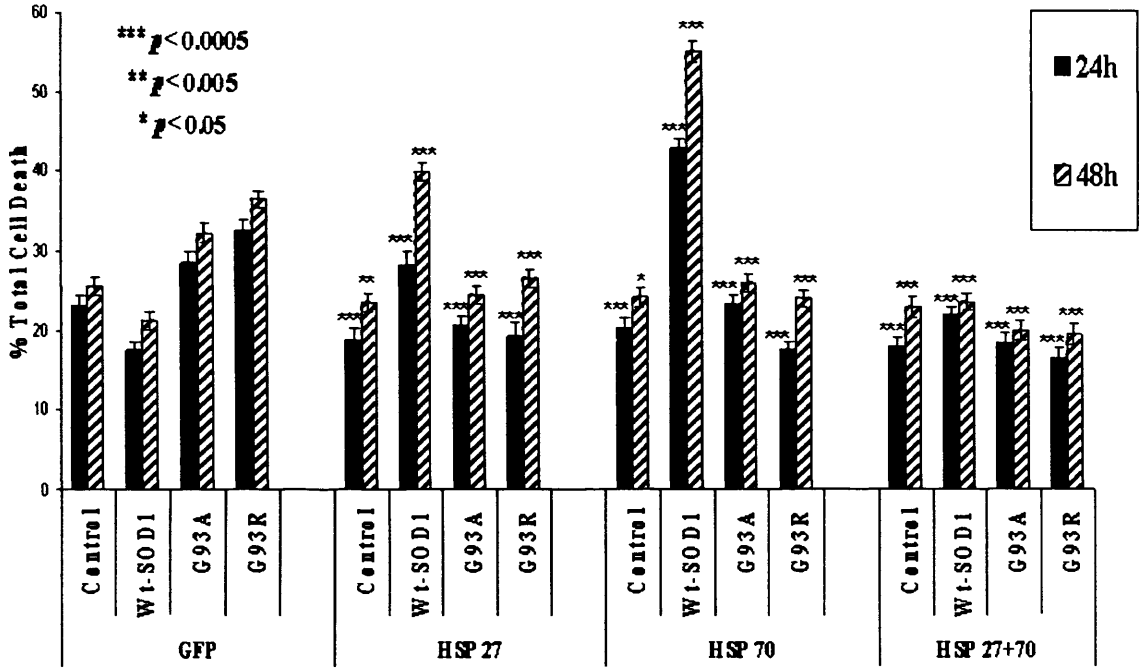



**Figure 4.8 Effect of camptothecin in SOD1 stable cells following Hsp infection**

**a.** ND7 cell death following 24h and 48h of 1μM camptothecin treatment after infection with HSV vectors expressing Hsps. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h and 48h 1μM camptothecin treatment of engineered ND7 cells expressing wt or mutant SOD1. Cells were infected with Hsp viruses 16h prior to stress. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=5. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p$<0.0005). *, **, *** indicates statistically significant difference between means, when comparing with the respective GFP virus infected cell line.

**Figure 4.8b**

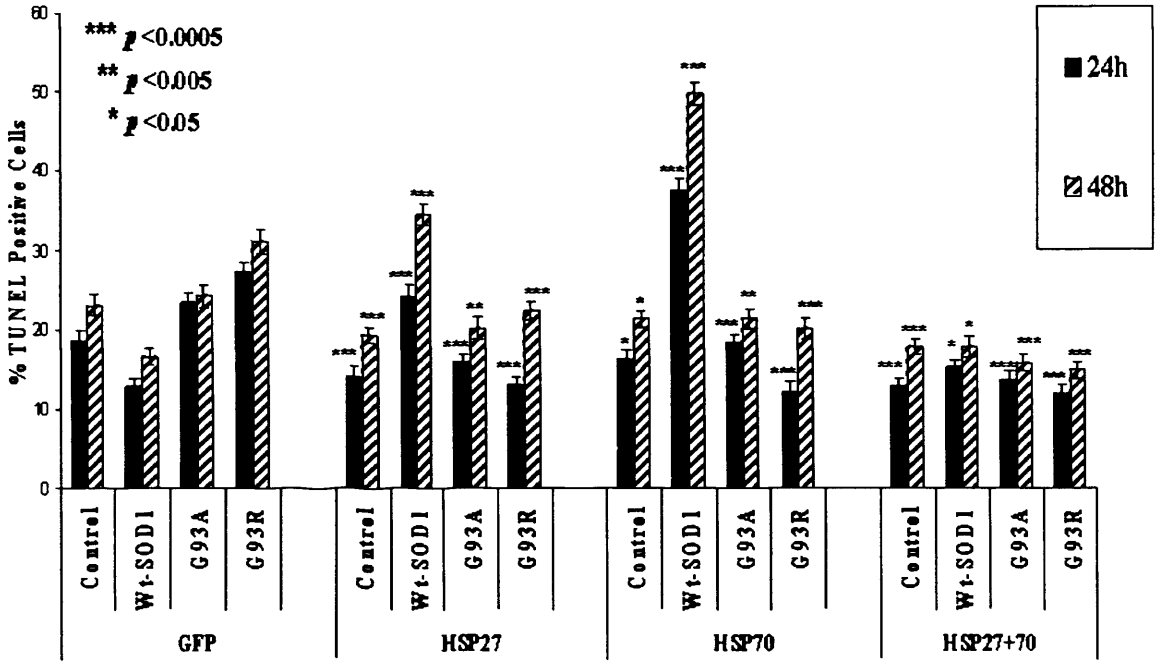
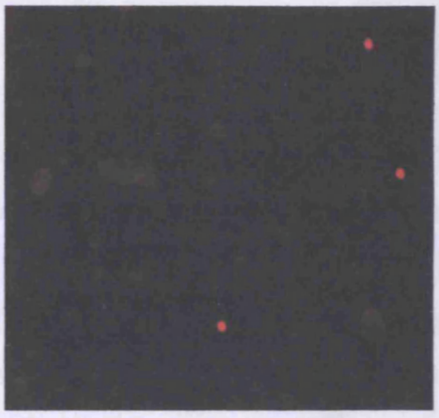
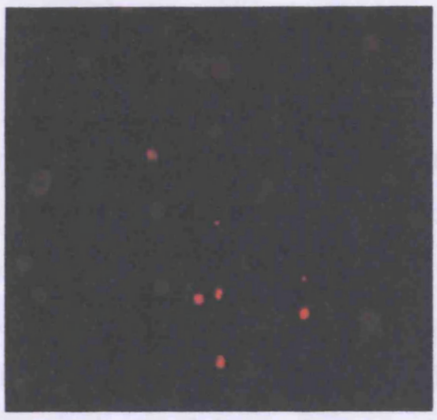



**Figure 4.8 Effect of camptothecin in SOD1 stable cells following Hsp infection**

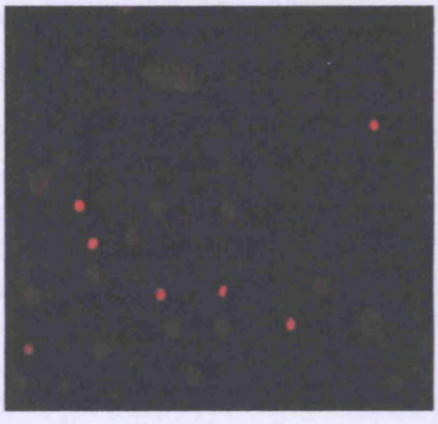
**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were infected with viruses to over-express Hsps or GFP 16h prior to 1μM camptothecin treatment for 24h and 48h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=3) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with the respective GFP virus infected cell line.



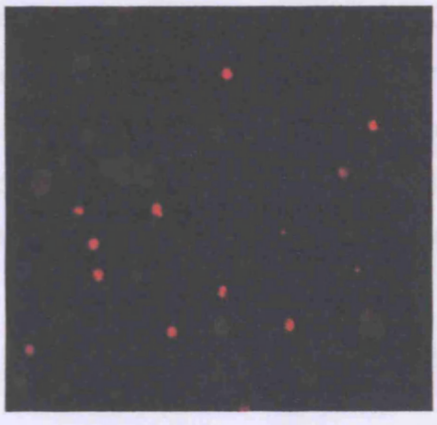
**(a)** Wt-SOD1 TUNEL +ve cells



**(b)** Control Vector TUNEL +ve cells



**(c)** G93A-SOD1 TUNEL +ve cells



**(d)** G93R SOD1 TUNEL +ve cells

**Figure 4.9 Images of ND7 cells positively stained with TdT-mediated dUTP nick end labelling (TUNEL).**

Rhodamine-conjugated nucleotides label the fragmented DNA in cells undergoing apoptosis, in this case on subjection to 24h serum removal. Blind counts of three random fields per slide were performed in order to determine the percentage of TUNEL positive cells adhered to the slide of **(a)** wt-SOD1 cells **(b)** control-vector cells **(c)** G93A and **(d)** G93R expressing cells. Only the cells that had a high intense fluorescence were counted as positive.

## 4.4 The Effect of Caspase Inhibitors and on SOD1 cells

The observed protective effects of wt-SOD1 cells on subjection to a range of stresses tested, including serum withdrawal (in the absence and presence of 1μM retinoic acid), staurosporine, IFN-γ, hydrogen peroxide, glutamate and camptothecin (described in Chapter 3), and the protective effects of Hsp27 and/or Hsp70 on the mutant SOD1 cells under all stresses (Chapter 4) in the described cellular model required further investigation. In order to investigate the protective effects of the Hsps further and begin to understand the mechanisms involved, a pan-caspase inhibitor (zVAD-FMK) and caspase-8 and caspase-9 specific inhibitors (z-IETD-FMK and z-LEHD-FMK, respectively) were employed to determine whether or not inhibition of all or parts of the caspase cascade could influence the survival of the SOD1 cell lines. The cells were subjected to a range of stresses as in Chapter 3, including serum withdrawal (in the absence and presence of 1μM retinoic acid), staurosporine, IFN-γ, hydrogen peroxide, glutamate and camptothecin. These experiments were extended to investigate whether protection conferred by the exogenous expression of Hsp27, Hsp70 or the Hsp27 and Hsp70 combination, involved modulation of the caspase cascade.

### 4.4.1 Serum Removal

All three caspase inhibitors produced significant effects on cell survival not only in the mutant-SOD1 cells but also in the wt-SOD1 cell lines following serum withdrawal, as illustrated in Figure 4.10. The pan caspase inhibitor z-VAD, as well as specific inhibitors of both caspase 8 and caspase 9 all significantly reduced cell death of the G93A and G93R SOD1 mutants (figure 4.10a) compared to respective controls ($p<0.0005$). On addition of zVAD death was reduced by approximately 38% and 57% for G93A and G93R mutants respectively compared to respective control ($p<0.0005$). Wt-SOD1 cells also exhibited a decrease in cell death on addition of zVAD with death being reduced by 36% compared to respective control ($p<0.0005$). Caspase 8 inhibitor reduced death by 32% and 52% for G93A and G93R respectively compared to respective control ($p<0.0005$). Caspase 9 inhibitor reduced death by approximately 31% and 52% for G93A and G93R respectively compared to respective control ($p<0.0005$). Wt-SOD1 death was reduced by approximately 30% and

29% on addition of caspase-8 and caspase-9 inhibitors respectively compared to control ($p<0.0005$).

Similar results were observed by assaying apoptosis by TUNEL staining ($p<0.0005$); (Figure 4.10b). G93A and G93R cells both exhibited a decrease in the number of TUNEL positive cells on addition of zVAD with number being reduced by approximately 46% and 63% respectively compared to control ($p<0.0005$). Caspase 8 and 9 inhibitors also reduced the numbers of TUNEL positive cells reducing the numbers by approximately 39% and 37% respectively for G93A and 56% and 57% respectively for G93R compared to control ($p<0.0005$). Addition of zVAD also reduced the number of TUNEL positive cells in the wt-SOD1 with numbers reduced by approximately 42% compared to control ($p<0.0005$). Addition of specific inhibitors for caspase 8 or caspase 9 to wt-SOD1 also brought about a reduction in the number of TUNEL positive cells with numbers decreasing by approximately 38% and 37% respectively compared to control ($p<0.0005$).

Figure 4.10a

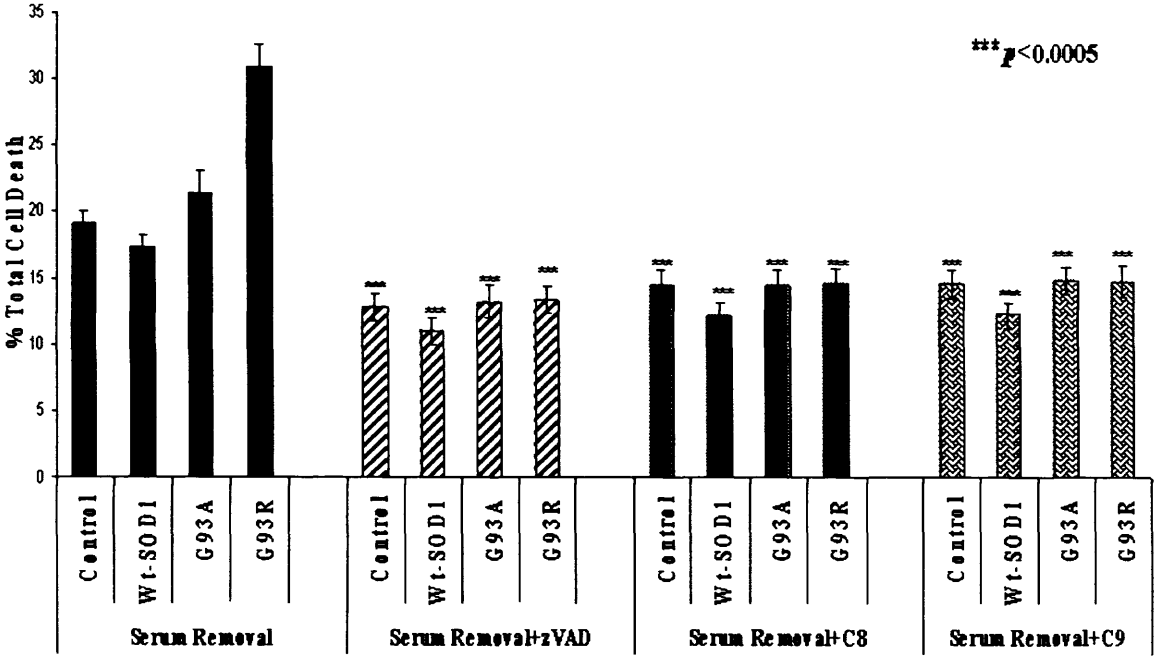



**Figure 4.10 Effect of caspase inhibitors in SOD1 stable cells following serum removal**

**a.** ND7 cell death following 24h serum removal or serum removal after incubation with caspase inhibitors, z-VAD, caspase-8 (C8) or caspase-9 (C9). The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of serum deprivation of engineered ND7 cells expressing wt or mutant SOD1. Cells were incubated with caspase inhibitors 1h prior to stress. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=5. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p<0.0005$). *, **, *** indicates statistically significant difference between means, when comparing with the respective control-vector, wt and G93A and G93R-SOD1 mutant control cells (cells exposed to stress of serum deprivation).

Figure 4.10b

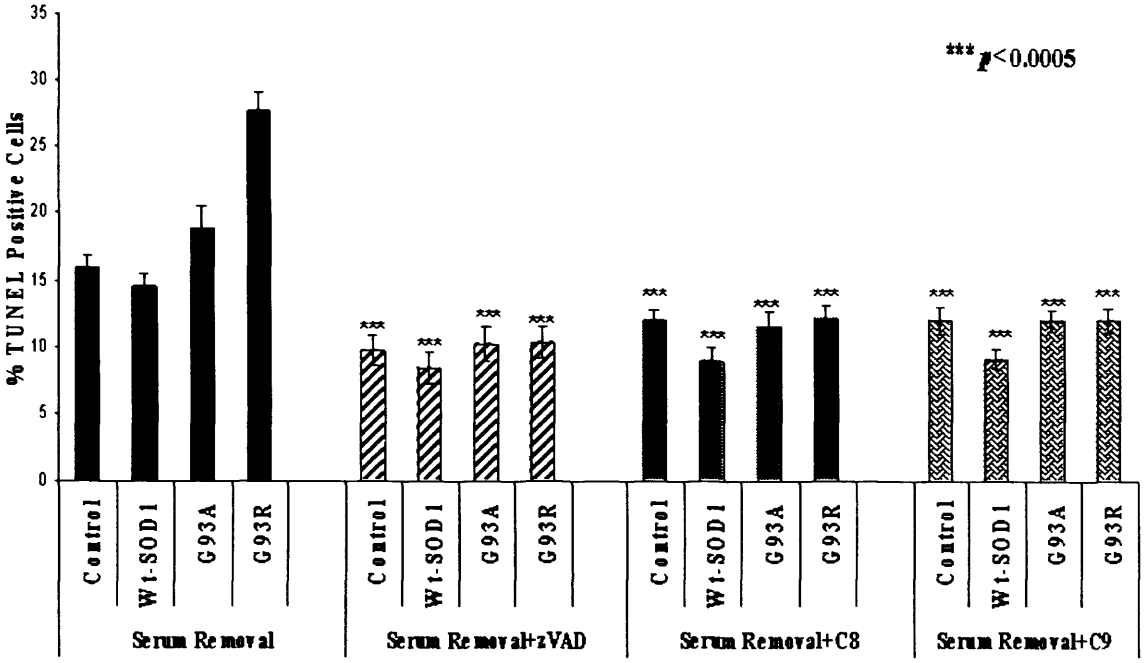



**Figure 4.10 Effect of caspase inhibitors in SOD1 stable cells following serum removal**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were incubated with caspase inhibitors 1h prior to treatment of serum deprivation for 24h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=3) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with the respective control-vector, wt and G93A and G93R-SOD1 mutant control cells.

### 4.4.2 Serum deprivation+ RA

Levels of cell death were reduced on addition of zVAD, caspase-8 and caspase-9 inhibitors across all cells (Figure 4.11a). Death was reduced in G93A mutant by approximately 52% on addition of zVAD compared to control ($p<0.0005$); (Figure 4.11a). On addition of caspase-8 inhibitor or caspase-9 inhibitor a reduction in cell death of approximately 50% was seen for both compared to control ($p<0.0005$). On addition of zVAD levels of cell death in the G93R cells was reduced by approximately 57% compared to control ($p<0.0005$). On addition of caspase-8 or caspase-9 inhibitor cell death in the G93R cells was reduced by approximately 55% in both instances compared to control ($p<0.0005$). Cell death was also lowered in wt-SOD1 cells on addition of zVAD with death being decreased by 33% compared to control ($p<0.0005$). On addition of caspase-8 or caspase-9 inhibitor cell death was reduced by 29% and 26% respectively compared to control ($p<0.0005$).

TUNEL analysis revealed similar findings with addition of zVAD reducing the number of TUNEL positive cells across all cell lines (Figure 4.11b). Wt-SOD1, G93A and G93R all exhibited reduction in the number of TUNEL positive cells with numbers reduced by approximately 42%, 57% and 61% respectively compared to respective control ($p<0.0005$); (Figure 4.11b). Both caspase-8 and caspase-9 inhibitors brought about a similar reduction with numbers of TUNEL positive cells. On addition of caspase-8 inhibitor to wt-SOD1, G93A and G93R cells the number of TUNEL positive cells were reduced by approximately 31%, 55% and 59% respectively compared to respective control ($p<0.0005$). On addition of caspase-9 inhibitor, the number of TUNEL positive cells were reduced by approximately 29%, 56% and 59% for wt-SOD1, G93A and G93R cells respectively compared to respective control ($p<0.0005$).

Figure 4.11a

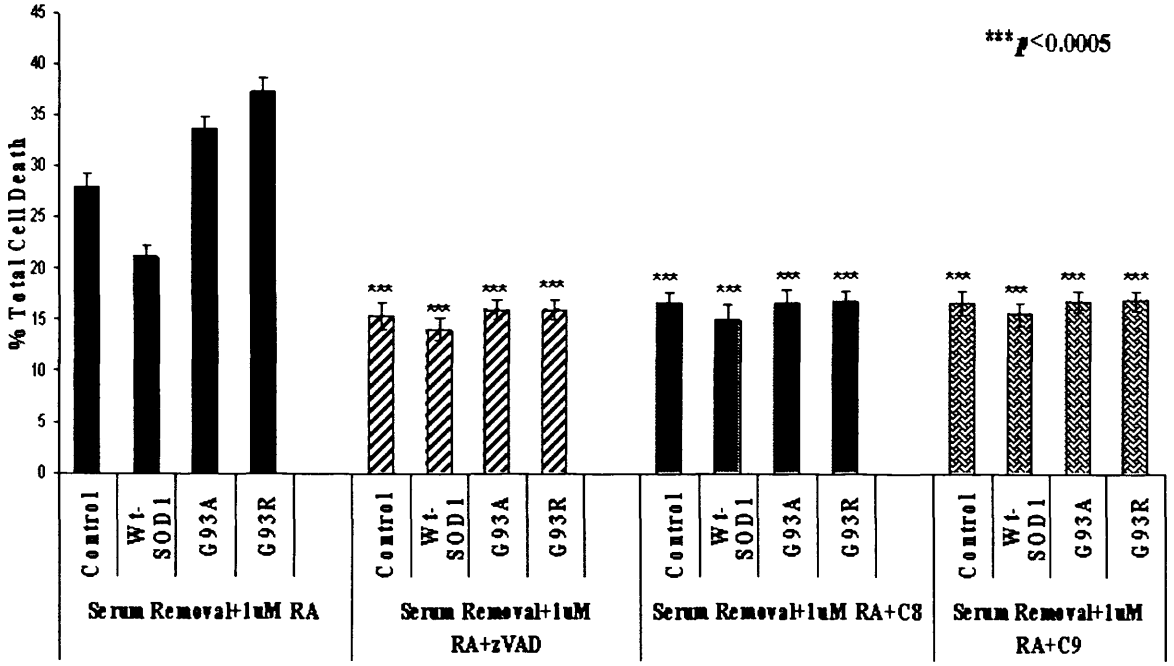



**Figure 4.11 Effect of caspase inhibitors in SOD1 stable cells following serum removal plus 1μM *all-trans* retinoic acid**

**a.** ND7 cell death following 24h of serum removal plus 1μM *all-trans* retinoic acid or serum removal plus 1μM *all-trans* retinoic acid after incubation with caspase inhibitors, z-VAD, caspase-8 (C8) or caspase-9 (C9). The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of serum deprivation plus 1μM *all-trans* retinoic acid of engineered ND7 cells expressing wt or mutant SOD1. Cells were incubated with caspase inhibitors 1h prior to stress. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=5. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p<0.0005$). *, **, *** indicates statistically significant difference between means, when comparing with the respective control-vector, wt and G93A and G93R-SOD1 mutant control cells (cells exposed to stress of serum deprivation plus 1μM *all-trans* retinoic acid).

Figure 4.11b

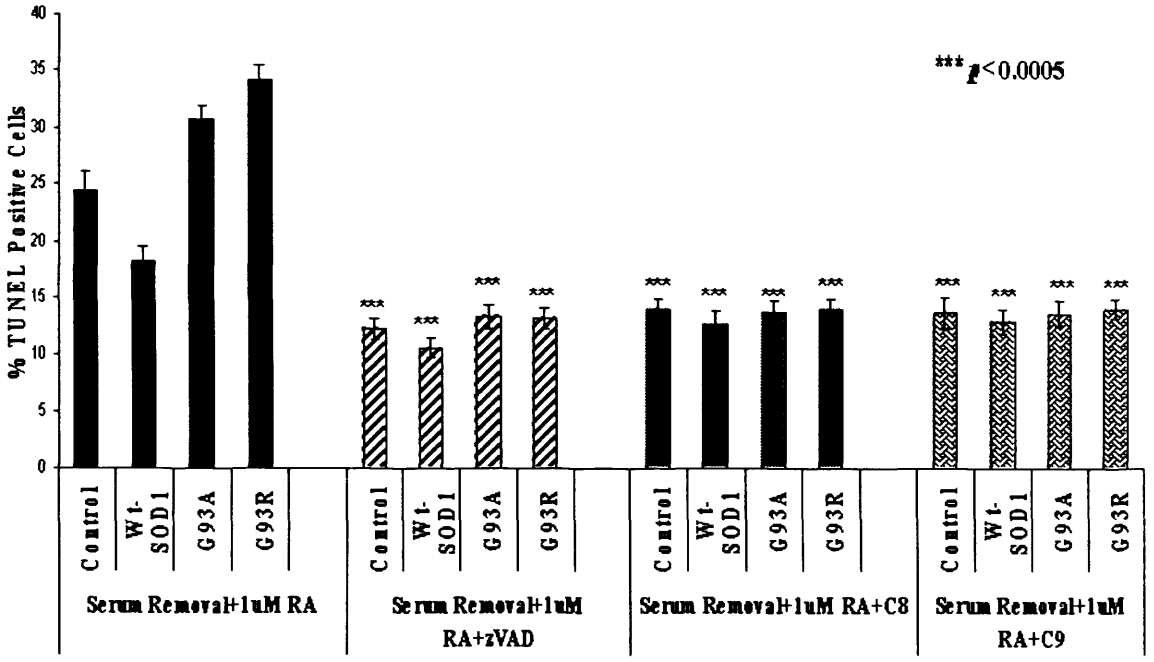



**Figure 4.11 Effect of caspase inhibitors in SOD1 stable cells following serum removal plus 1μM *all-trans* retinoic acid**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were incubated with caspase inhibitors 1h prior to treatment of serum deprivation plus 1μM *all-trans* retinoic acid for 24h. Bars represent mean $^{\pm}$ S.D. calculated for triplicate counts per sample (n=3) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with the respective control-vector, wt and G93A and G93R-SOD1 mutant control cells.

### 4.4.3 IFN-γ administration

Death was once again reduced across all cell lines on addition of pan caspase inhibitor zVAD and inhibitors of caspase-8 and caspase-9. Death was reduced in the G93A and G93R mutant on addition of zVAD by approximately 41% and 47% respectively compared to respective control ($p<0.0005$); (Figure 4.12a). Caspase-8 inhibitor reduced death by approximately 38% for G93A and 45% for G93R respectively compared to respective control ($p<0.0005$); (Figure 4.12a). Caspase-9 inhibitor reduced death by 37% for G93A and 43% for G93R compared to respective control ($p<0.0005$). Levels of cell death were reduced in wt-SOD1 cells by approximately 18% on addition of zVAD compared to control ($p<0.0005$). Inhibitors of caspase-8 and caspase-9 reduced levels of cell death in wt-SOD1 cells by approximately 13% in both instances compared to control ($p<0.0005$).

Similar results were obtained on TUNEL analysis (Figure 4.12b). Addition of zVAD reduced the number of TUNEL positive cells by approximately 44% and 54% in G93A and G93R cells respectively compared to respective control ($p<0.0005$); (Figure 4.12b). Inhibitors of caspase-8 and caspase-9 brought about a similar reduction with the number of TUNEL positive cells reduced by approximately 40% and 39% respectively for G93A cells and 51% and 48% respectively for G93R cells compared to respective control ($p<0.0005$). Wt-SOD1 cells also exhibited a similar reduction in the number of TUNEL positive cells on addition of zVAD with numbers reduced by approximately 23% compared to control ($p<0.0005$). Addition of inhibitors of caspase-8 or caspase-9 brought about a similar reduction in wt-SOD1 cells with numbers reduced by approximately 14% and 15% respectively compared to control ($p<0.005$).

Figure 4.12a

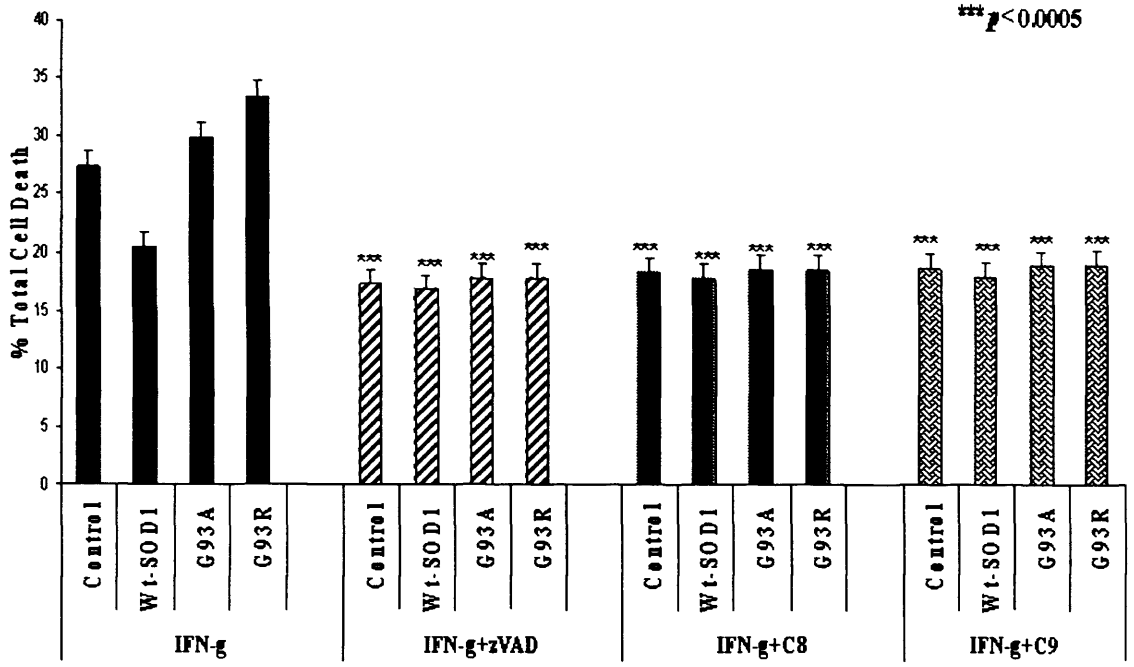



**Figure 4.12 Effect of caspase inhibitors in SOD1 stable cells following IFN-γ administration**

**a.** ND7 cell death following 24h of 50ng/ml IFN-γ or 50ng/ml IFN-γ after incubation with caspase inhibitors, z-VAD, caspase-8 (C8) or caspase-9 (C9). The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of IFN-γ treatment of engineered ND7 cells expressing wt or mutant SOD1. Cells were incubated with caspase inhibitors 1h prior to stress. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=5. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p$<0.0005). *, **, *** indicates statistically significant difference between means, when comparing with the respective control-vector, wt and G93A and G93R-SOD1 mutant control cells (cells exposed to stress of IFN-γ alone).

Figure 4.12b

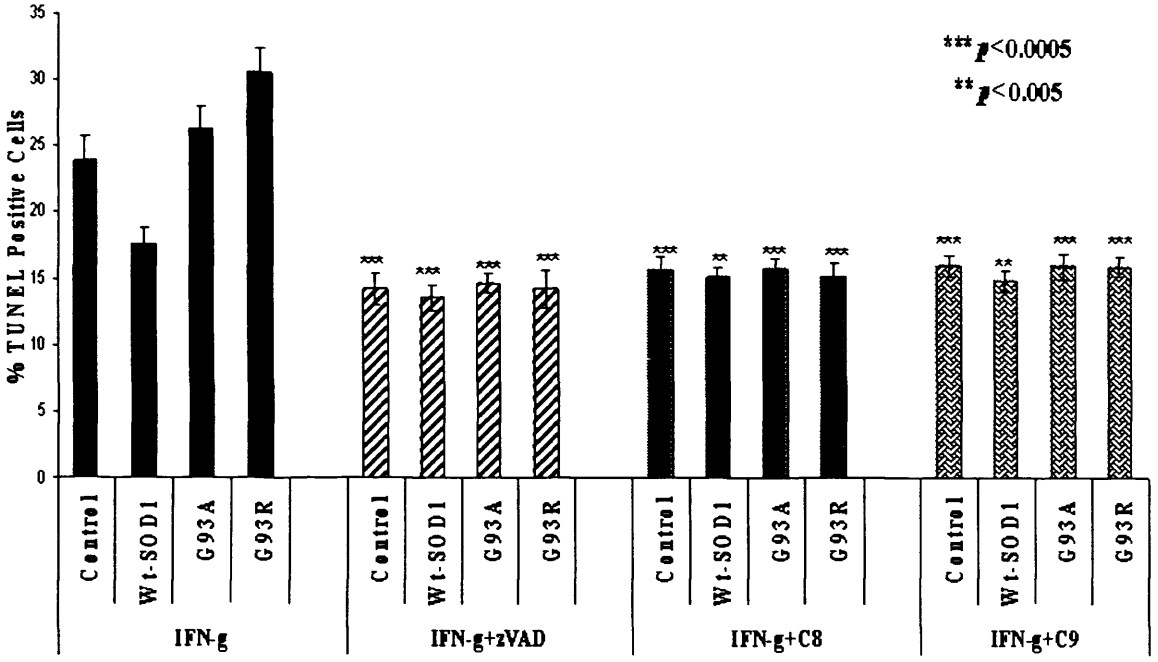



**Figure 4.12 Effect of caspase inhibitors in SOD1 stable cells following IFN-γ administration**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were incubated with caspase inhibitors 1h prior to treatment of 50ng/ml IFN-γ for 24h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=3) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with the respective control-vector, wt and G93A and G93R-SOD1 mutant control cells.

### 4.4.4 Staurosporine treatment

On addition of zVAD cell death was reduced across all cells. Level of cell death were reduced on addition of zVAD for G93A mutant by approximately 30% and 25% and for G93R mutant by 46% and 40% at 2h and 4h respectively compared to respective control ($p<0.0005$); (Figure 4.13a). Caspase-8 inhibitor reduced death in G93A cells by 28% and 21% and in G93R cells by 44% and 37% at 2h and 4h respectively compared to respective control ($p<0.0005$); (Figure 4.13a). Caspase-9 inhibitor also reduced death in both mutants with death in G93A cells being reduced by 27% and 21% and in G93R cells being reduced by 45% and 38% at 2h and 4h respectively compared to respective control ($p<0.0005$). Wt-SOD1 cells also showed a decrease in death following addition of zVAD or inhibitors of caspase-8 or caspase-9. Death was reduced in wt-SOD1 cells by 34% and 21% on addition of zVAD at 2h and 4h respectively compared to respective control cells($p<0.0005$). Inhibitor of caspase-8 reduced death in wt-SOD1 cells by approximately 23% and 14% at 2h and 4h respectively compared to respective control cells ($p<0.0005$). Inhibitor of caspase-9 reduced death in wt-SOD1 cells by approximately 20% and 14% at 2h and 4h respectively compared to respective control ($p<0.0005$).

TUNEL analysis revealed similar results with zVAD reducing the numbers of TUNEL positive cells in wt-SOD1, G93A and G93R cells. On addition of zVAD the number of TUNEL positive cells was reduced by approximately 34% and 26% for G93A cells and 51% and 43% for G93R cells at 2h and 4h respectively compared to respective control ($p<0.0005$); (Figure 4.13b). On addition of caspase-8 inhibitor the number of TUNEL positive cells was reduced by approximately 31% and 22% for G93A and 49% and 40% for G93R cells at 2h and 4h respectively compared to respective control ($p<0.0005$); (Figure 4.13b). On addition of caspase-9 inhibitor G93A and G93R cells exhibited a similar reduction in the number of TUNEL positive cells with numbers decreasing by approximately 29% and 22% for G93A and 49% and 41% for G93R cells at 2h and 4h respectively compared to respective control ($p<0.0005$). Wt-SOD1 cells exhibited a similar reduction in the number of TUNEL positive cells with zVAD addition bringing about a reduction of 40% and 22% at 2h and 4h respectively compared to control (p0.0005). On addition of caspase-8 inhibitor the number of TUNEL positive cells in the wt-SOD1 cells

was reduced by 28% and 16% at 2h and 4h respectively compared to control ($p<0.0005$). Addition of caspase-9 inhibitor to wt-SOD1 cells reduced the number of TUNEL positive cells by approximately 25% ($p<0.0005$) and 13% ($p<0.005$) at 2h and 4h respectively compared to control.

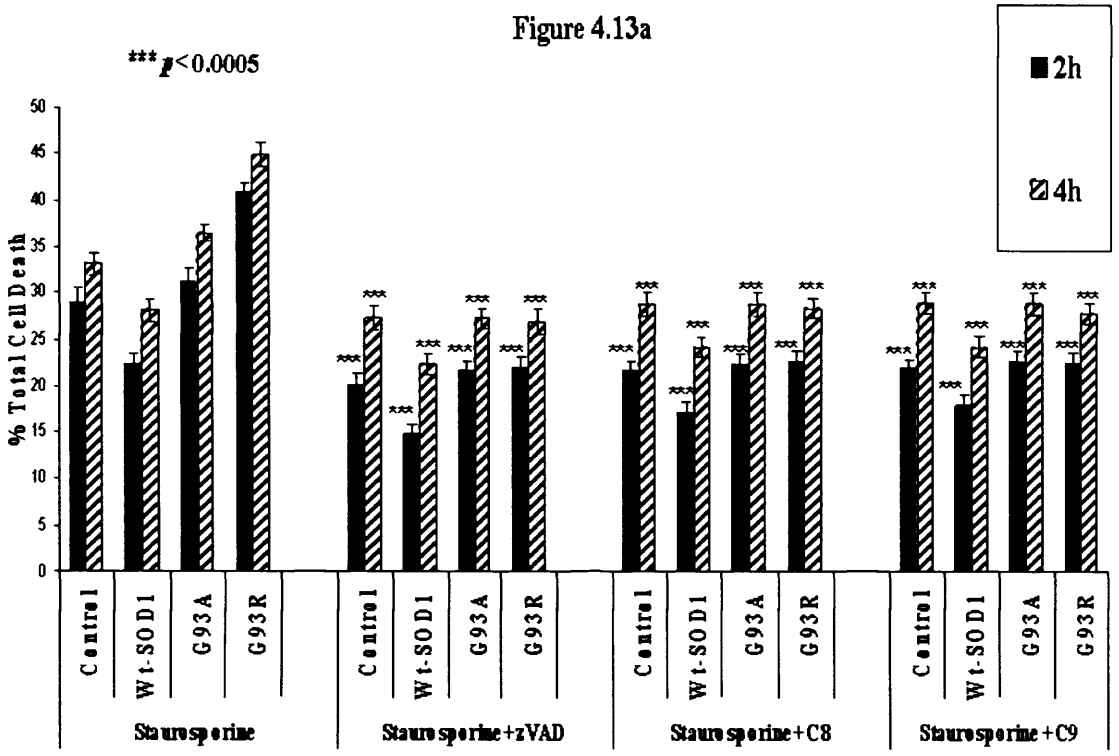



**Figure 4.13 Effect of caspase inhibitors in SOD1 stable cells following staurosporine treatment**

**a.** ND7 cell death following 24h of 1μM staurosporine treatment or 1μM staurosporine after incubation with caspase inhibitors, z-VAD, caspase-8 (C8) or caspase-9 (C9). The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of staurosporine treatment of engineered ND7 cells expressing wt or mutant SOD1. Cells were incubated with caspase inhibitors 1h prior to stress. Bars represent mean $^{+}$S.D. calculated for triplicate counts per sample (n), n=5. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p$<0.0005). *, **, *** indicates statistically significant difference between means, when comparing with the respective control-vector, wt and G93A and G93R-SOD1 mutant control cells (cells exposed to stress of staurosporine alone).

Figure 4.13b

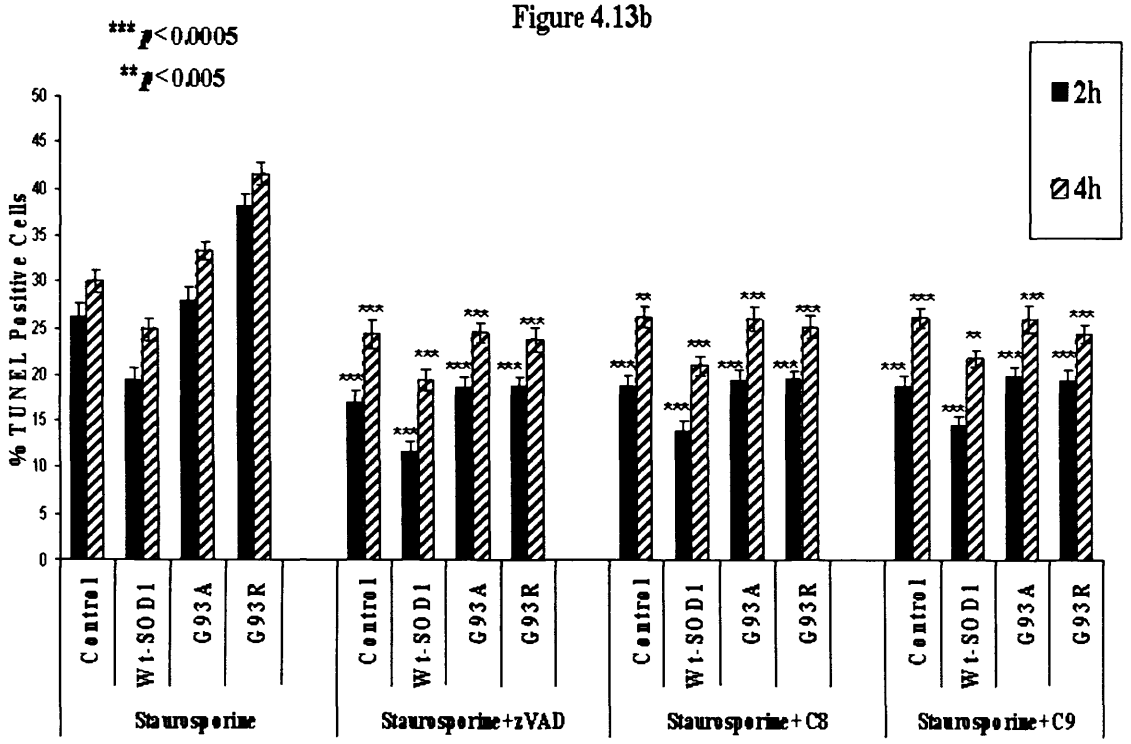



**Figure 4.13 Effect of caspase inhibitors in SOD1 stable cells following staurosporine treatment**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were incubated with caspase inhibitors 1h prior to treatment of 1μM staurosporine for 24h. Bars represent mean $^{\pm}$ S.D. calculated for triplicate counts per sample (n=3) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with the respective control-vector, wt and G93A and G93R-SOD1 mutant control cells.

### 4.4.5 Camptothecin administration

The pan-caspase inhibitor zVAD and inhibitors of caspase-8 and caspase-9 provided protection across all the cell lines. Addition of zVAD reduced death in the G93A and G93R mutant by approximately 39% and 50% respectively, compared to respective control ($p<0.0005$); (Figure 4.14a). Caspase-8 inhibitor reduced death in both G93A and G93R mutants by 36% and 47% respectively, compared to respective control ($p<0.0005$); (Figure 4.14a). Death was also reduced on addition of caspase-9 inhibitor with G93A cells seeing a 34% reduction and G93R cells a 46% reduction, compared to respective control ($p<0.0005$). In wt-SOD1 cells, zVAD reduced death by approximately 11% compared to control ($p<0.005$). On addition of caspase-8 or caspase-9 inhibitors death in wt-SOD1 cells was reduced by approximately 1% in both instances, compared to control.

TUNEL analysis revealed similar results with zVAD reducing the number of TUNEL positive cells in G93A and G93R by approximately 45% and 54% respectively compared to respective control ($p<0.0005$); (Figure 4.14b). On addition of caspase-8 inhibitor the number of TUNEL positive cells was reduced by approximately 41% and 52% for G93A and G93R respectively compared to respective control ($p<0.0005$); (Figure 4.14b). Caspase-9 inhibitor also reduced the number of TUNEL positive cells with numbers reduced by approximately 36% and 51% for G93A and G93R respectively compared to respective control ($p<0.0005$). Wt-SOD1 cells exhibited similar results with zVAD reducing the number of TUNEL positive cells by approximately 10% compared to control. Caspase-8 inhibitor or caspase-9 inhibitor both reduced the number of TUNEL positive cells in wt-SOD1 by approximately 1% and 3% respectively compared to control.

Figure 4.14a

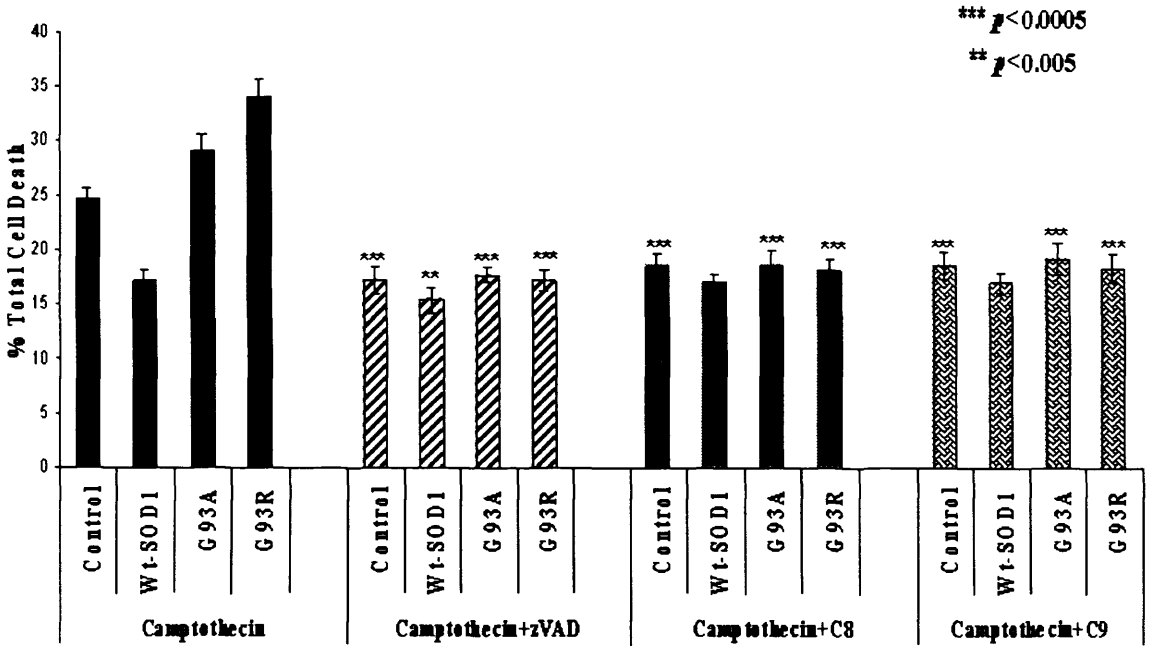



**Figure 4.14 Effect of caspase inhibitors in SOD1 stable cells following camptothecin administration**

**a.** ND7 cell death following 24h of 1μM camptothecin or 1μM camptothecin after incubation with caspase inhibitors, z-VAD, caspase-8 (C8) or caspase-9 (C9). The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of camptothecin treatment of engineered ND7 cells expressing wt or mutant SOD1. Cells were incubated with caspase inhibitors 1h prior to stress. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=5. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p$<0.0005). *, **, *** indicates statistically significant difference between means, when comparing with the respective control-vector, wt and G93A and G93R-SOD1 mutant control cells (cells exposed to stress of camptothecin alone).

Figure 4.14b

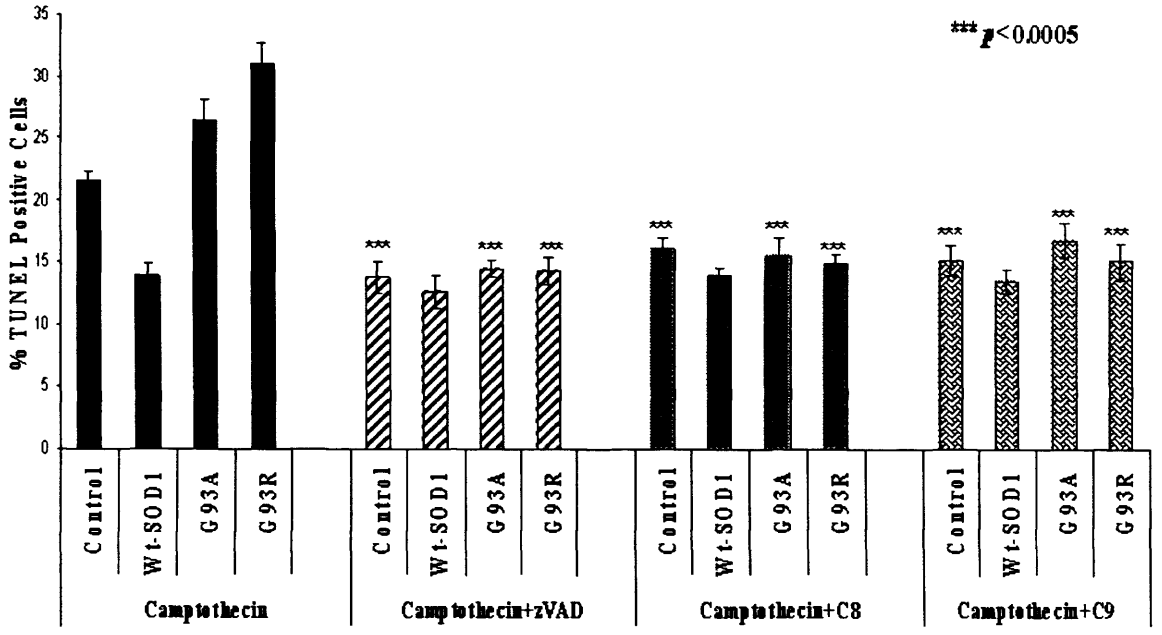



**Figure 4.14 Effect of caspase inhibitors in SOD1 stable cells following camptothecin administration**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were incubated with caspase inhibitors 1h prior to treatment of 1μM camptothecin for 24h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=3) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with the respective control-vector, wt and G93A and G93R-SOD1 mutant control cells.

## 4.5 The Effect of Hsp27 and/or Hsp70 plus Caspase Inhibitors on Wild Type and Mutant SOD1 Cells

Whilst all three caspase inhibitors produced significant protective effects on cell survival in the SOD1 cells following exposure to stress, discussed in section 4.4, with zVAD (the pan-caspase inhibitor) and specific inhibitors of both caspase-8 and caspase-9 suppressing cell death in the SOD1-mutant cells to the level observed in the control cell line, with the difference in cell death being statistically significant ($p<0.0005$). The degree of protection observed in these mutant cells following caspase inhibition was similar to that conferred by the exogenous expression of Hsp27 and/or Hsp70 in these cell lines. Cell death was assessed by both trypan blue exclusion assay (Figure 4.15a) and TUNEL analysis (Figure 4.15b). Interestingly, overexpression of Hsp27 and/or Hsp70 in the SOD1 cells in combination with the pan-caspase inhibitor zVAD, produced no additive protective effect on exposure to conditions of serum withdrawal compared infection with Hsp27 and/or Hsp70 in the absence of zVAD.

Similar results were also obtained in repeated experiments in which the percentage of apoptotic cells were assayed by TUNEL analysis with no synergy in reducing the number of apoptotic cells observed following the application of the pan-caspase inhibitor zVAD to the mutant-SOD1 cell lines that were over-expressing Hsp27 and/or Hsp70.

G93A and G93R mutant cells infected with Hsp27 and subjected to serum withdrawal, exhibited an 11% ($p<0.005$) and 36% ($p<0.0005$) reduction in cell death respectively compared to respective GFP infected control (Figure 4.15a). On infection of G93A and G93R mutant cells with Hsp70 death was reduced by 7% ($p<0.05$) and 44% ($p<0.0005$) respectively compared to respective GFP infected control (Figure 4.15a). The combinational infection of Hsp27 and Hsp70 was the most effective in the reduction of death of both G93A and G93R mutant cells. On dual infection of G93A mutant cells a reduction of approximately 21% was observed and an approximately 48% reduction in the G93R mutant cells compared to respective GFP control ($p<0.0005$). On addition of zVAD, the mutant G93A and G93R cells saw a reduction of approximately 40% and 61% respectively

compared to respective GFP control ($p<0.0005$). The combination of Hsp27 and zVAD did not provide an additive effect with G93A cells exhibiting an 8% and G93R cells a 33% ($p<0.0005$) reduction in death respectively compared to respective GFP control. The combination of Hsp70 and zVAD was also unsuccessful in providing an addition protective effect with G93A cells seeing a rise of almost 1% and the G93R cells a reduction of approximately 38% ($p<0.0005$) compared to respective GFP control. The dual infection of Hsp27 and Hsp70 with zVAD was also not as effective in reducing death as either the dual infection of Hsp27 and Hsp70 alone or zVAD alone.

TUNEL analysis revealed similar results with Hsp27 reducing the number of TUNEL positive cells by approximately 15% ($p<0.05$) and 35% ($p<0.0005$) for G93A and G93R respectively compared to respective control (Figure 4.15b). Hsp70 also resulted in a reduction in the number of TUNEL positive cells with the numbers reduced by approximately 10% and 45% ($p<0.0005$) for G93A and G93R respectively compared to respective control (Figure 4.15b). The dual combination of Hsp27 and Hsp70 reduced the number of TUNEL positive cells by approximately 22% and 47% for G93A and G93R cells respectively compared to respective control ($p<0.0005$). The combination of Hsp27 and zVAD was not as effective as either singly and reduced the number of TUNEL positive cells by approximately 10% and 32% ($p<0.0005$) for G93A and G93R respectively compared to respective control. The combination of Hsp70 and zVAD was also not as effective as either singly and only reduced the number of TUNEL positive cells by approximately 2% and 37% ($p<0.0005$) for G93A and G93R respectively compared to respective control. The combination of Hsp27 and Hsp70 along with zVAD was only slightly more effective than either Hsp27 or Hsp70 with zVAD but still not as effective as the single respective additions with the number of TUNEL positive cells reduced by approximately 12% ($p<0.005$) and 43% ($p<0.0005$) for G93A and G93R respectively compared to respective control. Wt-SOD1 cells exhibited an increase in the number of TUNEL positive cells on infection with Hsp27 or Hsp70 with the number increasing by approximately 12% and 113% ($p<0.0005$) respectively compared to control. The dual infection with Hsp27 and Hsp70 brought the levels down toward control levels with the number of TUNEL positive cells reduced by approximately 2% compared to control. The combination of Hsp27+zVAD or Hsp70+zVAD increased the number of TUNEL positive cells by approximately 18% and

121% ($p<0.0005$) respectively compared to control. The combination of Hsp27 and Hsp70 with zVAD resulted in an increase in the number of TUNEL positive cells by approximately 11% compared to control. Therefore, from the results obtained it appears that the Hsps act via inhibition of caspases.

Figure 4.15a

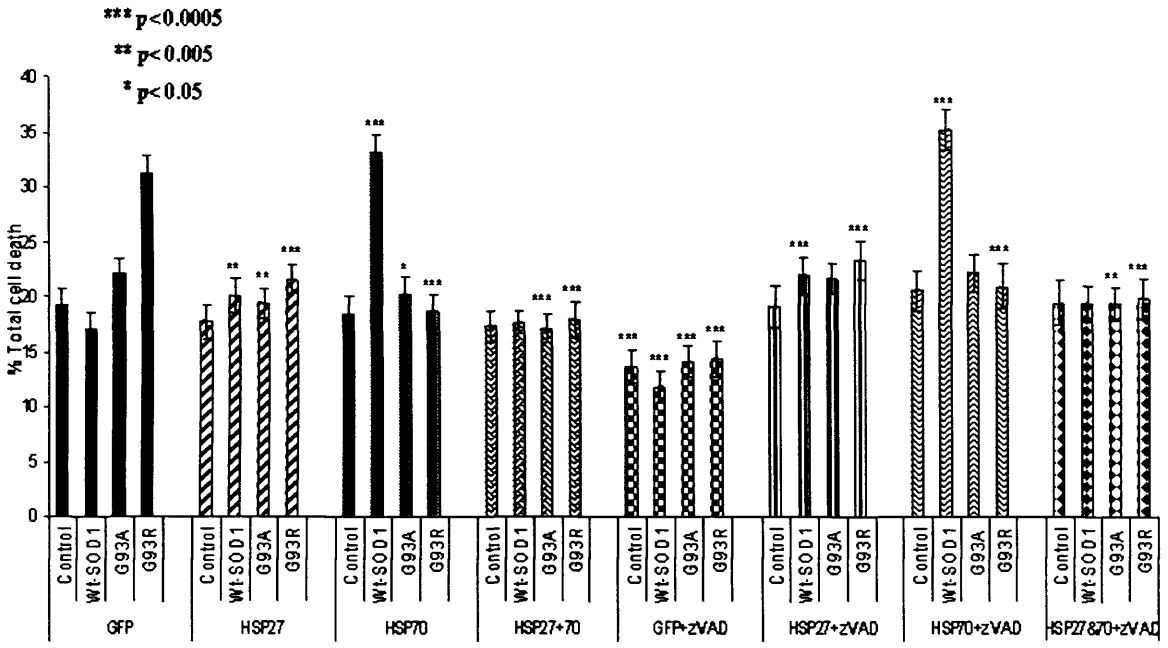



**Figure 4.15 The dual effect of Hsp's and caspase inhibitors in SOD1 stable cells following serum removal**

**a.** ND7 cell death following 24h serum removal after infection with either GFP, Hsp27, Hsp70 or Hsp27 and Hsp70 virus plus incubation with caspase inhibitor z-VAD. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of serum deprivation of engineered ND7 cells expressing wt or mutant SOD1. Cells were incubated with caspase inhibitors 1h prior to stress. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=5. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p<0.0005$). *, **, *** indicates statistically significant difference between means, when comparing with the respective GFP infected control-vector, wt, G93A or G93R-SOD1 mutant control cells.

**Figure 4.15b**

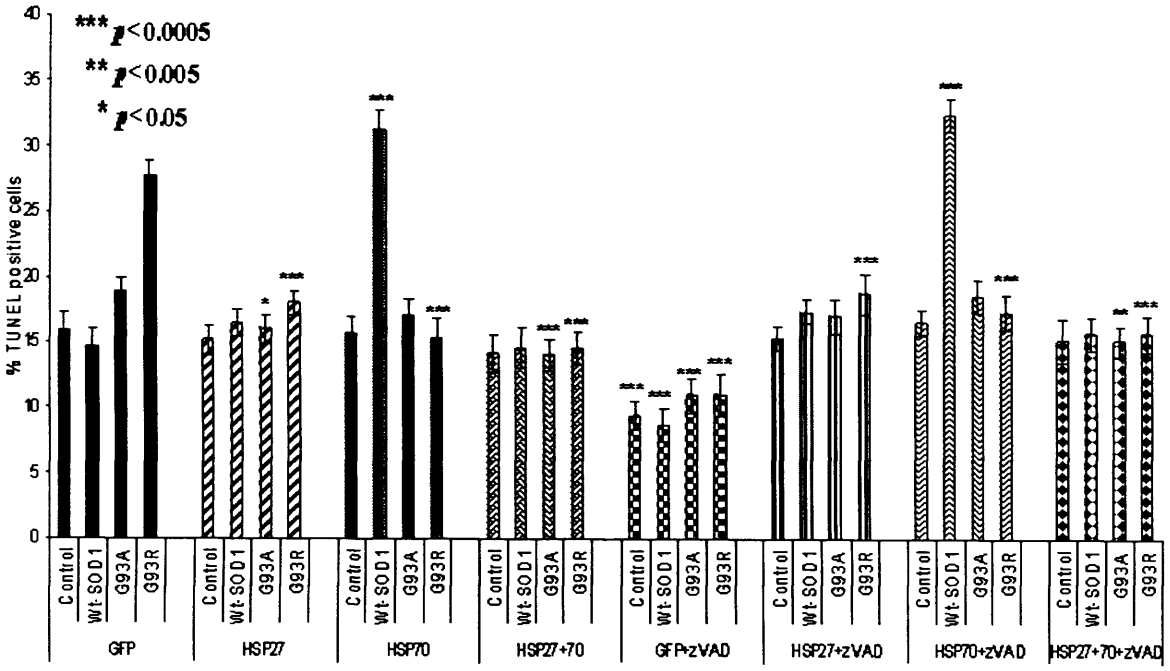



**Figure 4.15 The dual effect of Hsp's and caspase inhibitors in SOD1 stable cells following serum removal**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were incubated with caspase inhibitors 1h prior to treatment of serum deprivation for 24h. Bars represent mean $^{\pm}$ S.D. calculated for triplicate counts per sample (n=3) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with the respective GFP infected control-vector, wt, G93A or G93R-SOD1 mutant control cells.

## 4.6 Responses of the SOD1 cell lines to Stress following addition of ApoE2, ApoE3 or ApoE4 alleles

Apolipoprotein E (ApoE for the protein, APOE for the gene) is a widely distributed, well-characterized cholesterol transport protein that circulates in the plasma following its synthesis in the liver, spleen, and kidneys. It is the major apolipoprotein in the central nervous system (CNS) (Pitas et al., 1987), where it is synthesized by glia, macrophages (Boyles et al., 1985), and also neurons (Han et al., 1994). Additionally, it is also found in the peripheral nervous system (PNS), where it is produced by non-myelinating Schwann cells, ganglionic satellite cells, and macrophages (Boyles et al., 1985). In humans, ApoE exists in 3 common isoforms: ε2, ε3, and ε4, which differ from one another at amino acid positions 112 and 158. Isoform ε3 has a cysteine at 112, and an arginine at 158 whereas ε2 has cysteines at both positions and ε4 has arginines at both positions. The absolute frequency of these different isoforms is dependent on the population being studied, however the relative frequency is always similar, with ε3 being much more common than ε2 or ε4.

### 4.6.1 Motor Neuron Disease and ALS

Despite four large studies, the association between APOE and motor neuron disease (MND) and/or ALS as yet remains controversial. Mui and co-workers (1995) were the first to investigate this relationship. They examined 170 patients with undefined "ALS" of which 72 had sporadic disease, 77 had a family history with unknown genetic predisposition, and 21 had a family history of a mutation in superoxide dismutase (SOD1). The ALS patients were compared with 1209 historical age-matched controls (Ordovas et al., 1987) and 60 ALS-free siblings or spouses of the familial group. There was no difference observed in APOE allele frequency in any of the ALS groups compared to controls. In addition, no difference was found in ALS onset age or disease duration between patients with an ε4 allele and those without. This study along with other ALS studies to date, all used a small sample size. Only the patients with familial ALS were used in the analysis of disease duration, and only 43 of 72 patients with sporadic disease were included in the assay for onset age but the

loss of the other 29 patients was not explained. Most seriously of all, this study did not define the criteria for diagnosing ALS.

Work carried out by Al-Chalabi and colleagues (1996) obtained different results. In this study, APOE genotyping was carried out on 123 patients with MND (diagnosed by two consulting neurologists) and 121 geographically similar disease-free controls. The frequency of APOE4 genotypes in the MND patient group was not found to be significantly different from that in their controls though there was a trend toward the MND population having a higher APOE4 genotype frequency (27.6%) than the controls (21.5%). In addition, subdivision of the patients with MND into those with limb-onset disease and those with bulbar-onset disease, revealed the latter group had a significantly higher frequency of APOE4 genotypes than the controls (42.4%; P= .02). On plotting the probability of survival as a function of time through Kaplan-Meier analysis, it was discovered that patients with ε4 alleles had a slightly greater rate of death than patients without. Even though this difference did not reach statistical significance, it converted to a median survival of 35 months for patients with an ε4 allele and 49 months for those without which would seem clinically important.

This study conducted by Al-Chalabi et al. (1996) also suffers from small sample size, especially in analyses of subgroups such as patients with limb onset and bulbar onset; the latter group included only 33 patients. In addition, it fails to define the clinical or electrical criteria used in making the diagnosis of MND, and does not specify the etiology of this condition in its patient group apart from saying they did not have dementia, spinal muscular atrophy, or Kennedy syndrome.

The findings of Al-Chalabi et al (1996) were confirmed and extended by work carried out by Moulard and colleagues (1996) who examined 130 patients with sporadic ALS, defined in accordance with the El Escorial criteria (Brooks. 1994), and 675 geographically similar historical controls (Schachter et al., 1994; Luc et al., 1994). No significant difference was observed in APOE genotype frequency between patients with ALS and controls. However there was a trend toward patients with ALS having a lower frequency of ε2-containing genotypes (8.5%) than the control population (15%). In this study no

relationship was found between ALS onset age and allele type. Also no difference was found in ALS duration between patients with and without ε4 alleles. Interestingly, however a statistically significant difference in disease duration was found to occur between patients with and without ε2 alleles with those with ε2 alleles exhibiting longer survival. In addition, a statistically significant relationship emerged between ALS onset location and allele type, with patients with ε2 alleles more likely to halve limb-onset disease, whereas patients with ε4 were more likely to have bulbar-onset disease. Among the patients with limb onset, those possessing ε2 alleles were found to survive significantly longer than those without. Among the patients with bulbar onset, those possessing ε4 alleles exhibited disease symptoms at a significantly earlier age than those without.

Although studies have examined the protective effects of ApoE2 over ApoE4, as yet it remains controversial as to whether there exists an association between APOE and ALS. In the present project, APOE alleles were utilised in order to assess whether there exists a difference in relationship between the APOE alleles in the level of protection conferred against various stresses applied in an *in vitro* system in which wt-SOD1 or the disease-associated mutants, G93A and G93R-mutant SOD1, are stably over-expressed. Cells were subjected to a wide range of stresses such as serum removal, serum removal plus 1μM *all-trans* retinoic acid, IFN-γ treatment, staurosporine administration and camptothecin addition, in order to compare their response to these distinct death stimuli at multiple time points, in the presence or absence APOE alleles – APOE2, APOE3 or APOE4.

The following section briefly describes the creation of the cell lines and the method of isolation of the ApoE protein, followed by a description of the results obtained following experimentation into the protective effect of APOE alleles - APOE2, APOE3 and APOE4 - in this model system, on exposure to each death stimulus. The findings for the various stresses are presented below the corresponding stress and cell death was assessed by trypan blue exclusion assay and TUNEL analysis. Finally, the significance of the findings is discussed in the discussion for the chapter.

### 4.6.2 Creation of APOE cells and Isolation of APOE

The ApoE protein isoforms were a gift from Dr B.S. Thilakawardhana, Royal Free Hospital, UCL. Briefly CHO (Chinese Hamster Ovary) cells – $CHO^{dhfr-}$ cells were cultured in Iscove's modified Dulbecco's medium with 10% dialyzed fetal bovine serum supplemented with 2mM glutaMAX, 100μM hypoxanthine, 16μM thymidine, and 1% non-essential amino acids (see Tagalakis et al., 2001).

Human APOE2 cDNA was cloned into the expression vector p7055 (see Miloux et al., 1994), which contains the selectable DHFR (dihydrofolate reductase) gene. Approximately 6μg of p7055.E2 was complexed with cationic liposomes to transfect $CHO^{dhfr-}$ cells ($2x10^6$). Cells were grown for 2-3 weeks in Iscove's selection medium, which lacks hypoxanthine and thymidine, clones of CHO-E2 were isolated by limited dilution; Clones of CHO-E3 and CHO-E4 were produced similarly. Subsequently the medium of the clones was screened for ApoE by ELISA and the four most productive clones expanded for each APOE cell lines (see Tagalakis et al., 2001). The ApoE secreted into the culture medium was measured by two-antibody sandwich ELISA, using a goat polyclonal anti-human ApoE for capture and biotinylated goat polyclonal anti-human ApoE for detection (see Tagalakis et al., 2001).

## 4.7 Responses of Cell Lines to Several Death-inducing Stimuli following addition of ApoE

### 4.7.1 Serum Removal

G93A and G93R mutant SOD1 expressing cells exposed to serum removal and addition 50ng of ApoE-4 exhibited an increase in total cell death by 36% and 39% respectively compared to respective cell control ($p<0.0005$); (Figure 4.16a). ApoE3 treated G93A and G93R mutant cells showed a reduction in total cell death of 11% and 21% respectively compared to respective control cells ($p<0.0005$); (Figure 4.16a). ApoE2 treated mutant cells showed an even greater reduction in cell death with death being reduced by approximately

18% and 33% for G93A and G93R cells respectively compared to respective control ($p<0.0005$). Wt-SOD1 expressing cells also exhibited an increase in cell death on addition of ApoE4 with total cell death rising by approximately 22% compared to control ($p<0.0005$). ApoE3 addition led to reduction in total cell death of wt-SOD1 expressing cells by approximately 9% compared to control. Addition of ApoE2 was most effective in reducing cell death just as in case of mutants with cell death being reduced by 18% compared to control ($p<0.0005$).

TUNEL analysis brought about similar results further confirming the protective effect of ApoE2 and ApoE3 over ApoE4. G93A and G93R mutant expressing cells each showed an increase in the number of TUNEL positive cells on addition of ApoE4 with an increase of 32% and 41% for G93A and G93R respectively compared to respective control ($p<0.0005$); (Figure 4.16b). ApoE3 and ApoE2 both brought about a reduction in number of TUNEL positive cells with ApoE3 reducing the number of TUNEL positive cells in the case of G93A by approximately 12% compared to control ($p<0.05$); (Figure 4.16b). In the case of G93R, ApoE3 brought about a reduction in TUNEL positive cells by approximately 16% compared to control ($p<0.0005$). On addition of ApoE2 apoptotic death in both mutants was reduced further with a reduction of 24% and 34% being observed for G93A and G93R respectively compared to respective control ($p<0.0005$). Wt-SOD1 cells showed a similar trend to the mutants with ApoE4 bringing about an increase of in the number of TUNEL positive cells by approximately 24% compared to control ($p<0.0005$). ApoE3 addition, as with mutants, showed a protective effect in wt-SOD1 cells bringing about a reduction in the number of TUNEL positive cells by approximately 9% compared to control. ApoE2 addition brought the number of TUNEL positive wt-SOD1 cells down even further with a reduction of 22% being observed compared to control ($p<0.005$).

**Figure 4.16a**

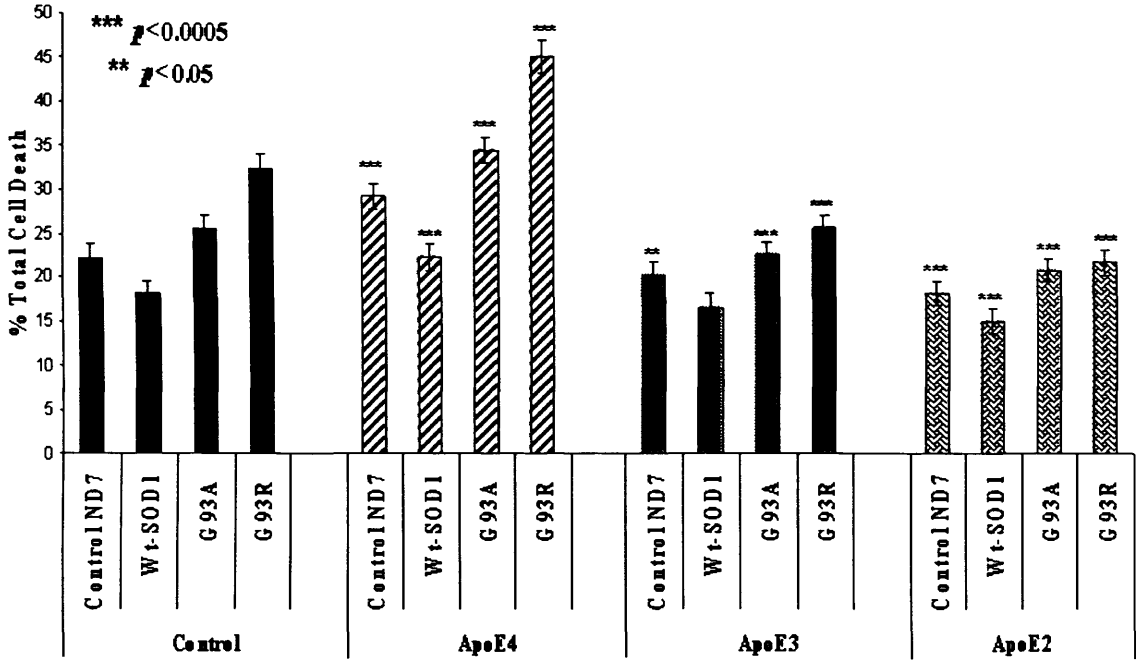



**Figure 4.16 Effect of ApoE isoforms on SOD1 stable cells following serum removal**

**a.** ND7 cell death following 24h of serum removal or serum removal after incubation with 50ng/ml Apolipoprotein-E (ApoE), ApoE4, ApoE3 or ApoE2. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of serum deprivation of engineered ND7 cells expressing wt or mutant SOD1. Cells were incubated with ApoE isoforms 1h prior to stress. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=5. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p$<0.0005). *, **, *** indicates statistically significant difference between means, when comparing with the respective control-vector, wt and G93A and G93R-SOD1 mutant control cells (cells treated with control solution obtained from control cells).

**Figure 4.16b**

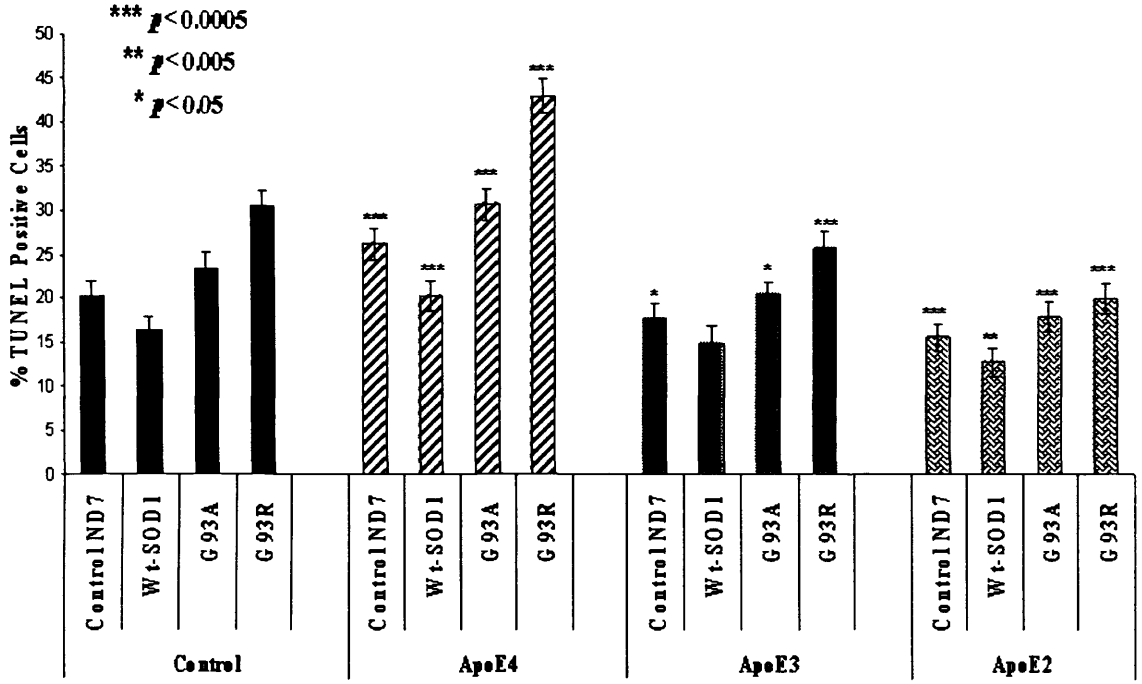



**Figure 4.16 Effect of ApoE isoforms on SOD1 stable cells following serum removal**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were incubated with ApoE protein 1h prior to treatment of serum deprivation for 24h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=3) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with the respective control-vector, wt and G93A and G93R-SOD1 mutant control cells.

### 4.7.2 Serum Removal +RA

G93A and G93R mutant SOD1 cells showed a similar increase when subjected to serum removal plus 1μM retinoic acid on addition of ApoE4. Total cell death increased by approximately 28% and 31% for G93A and G93R respectively compared to respective control ($p<0.0005$); (Figure 4.17a). On addition of ApoE3 death was reduced in both instances with death decreasing by approximately 18% and 25% for G93A and G93R respectively compared to respective control ($p<0.0005$); (Figure 4.17a). Addition of ApoE2 reduced death even further with death being reduced by approximately 36% and 50% for G93A and G93R respectively compared to respective control ($p<0.0005$). Wt-SOD1 cells also exhibited a similar trend to the mutants. Total cell death in the wt-SOD1 cells increased by approximately 12% on addition of ApoE4 compared to control ($p<0.05$). On addition of ApoE3 death was reduced as with mutants in wt-SOD1 cells with death decreasing by approximately 11% compared to control. Total cell death of wt-SOD1 cells was reduced the most by addition of ApoE2 with death being reduced by approximately 25% compared to control ($p<0.0005$).

TUNEL analysis revealed similar results with the number of TUNEL positive cells in both G93A and G93R mutant cells increasing by approximately 20% and 24% respectively on addition of ApoE4 compared to respective control ($p<0.0005$); (Figure 4.17b). On addition of ApoE3 number of TUNEL positive cells was reduced in both mutants by approximately 19% and 25% for G93A and G93R respectively compared to respective control ($p<0.0005$); (Figure 4.17b). The number of TUNEL positive cells was reduced even further by approximately 33% and 45% for G93A and G93R respectively on addition of ApoE2 compared to respective control ($p<0.0005$). Wt-SOD1 cells showed a similar trend to the mutants with the number of TUNEL positive cells increasing by approximately 16% on addition of ApoE4 compared to control ($p<0.005$). Addition of ApoE3 resulted in a reduction in the number of TUNEL positive cells with death decreasing by approximately 15% compared to control ($p<0.005$). Once again ApoE2 was the most effective in reducing to the number of TUNEL positive cells with wt-SOD1 cells exhibiting a 27% reduction in the number of TUNEL positive cells compared to control ($p<0.0005$).

Figure 4.17a

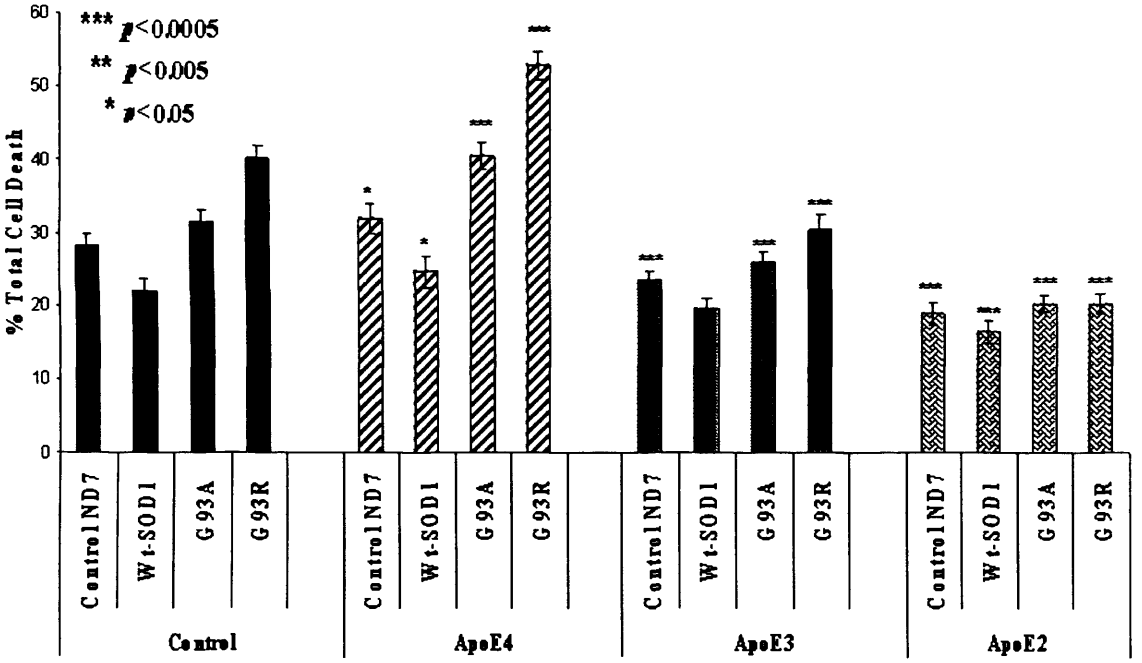



**Figure 4.17 Effect of ApoE isoforms on SOD1 stable cells following serum removal plus 1μM *all-trans* retinoic acid**

**a.** ND7 cell death following 24h of serum removal plus 1μM *all-trans* retinoic acid or serum removal plus 1μM *all-trans* retinoic acid after incubation with 50ng/ml Apolipoprotein-E (ApoE), ApoE4, ApoE3 or ApoE2. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of serum deprivation plus 1μM *all-trans* retinoic acid of engineered ND7 cells expressing wt or mutant SOD1. Cells were incubated with ApoE isoforms 1h prior to stress. Bars represent mean $\pm$S.D. calculated for triplicate counts per sample (n), n=5. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p<0.0005$). *, **, *** indicates statistically significant difference between means, when comparing with the respective control-vector, wt and G93A and G93R-SOD1 mutant control cells (cells treated with control solution obtained from control cells).

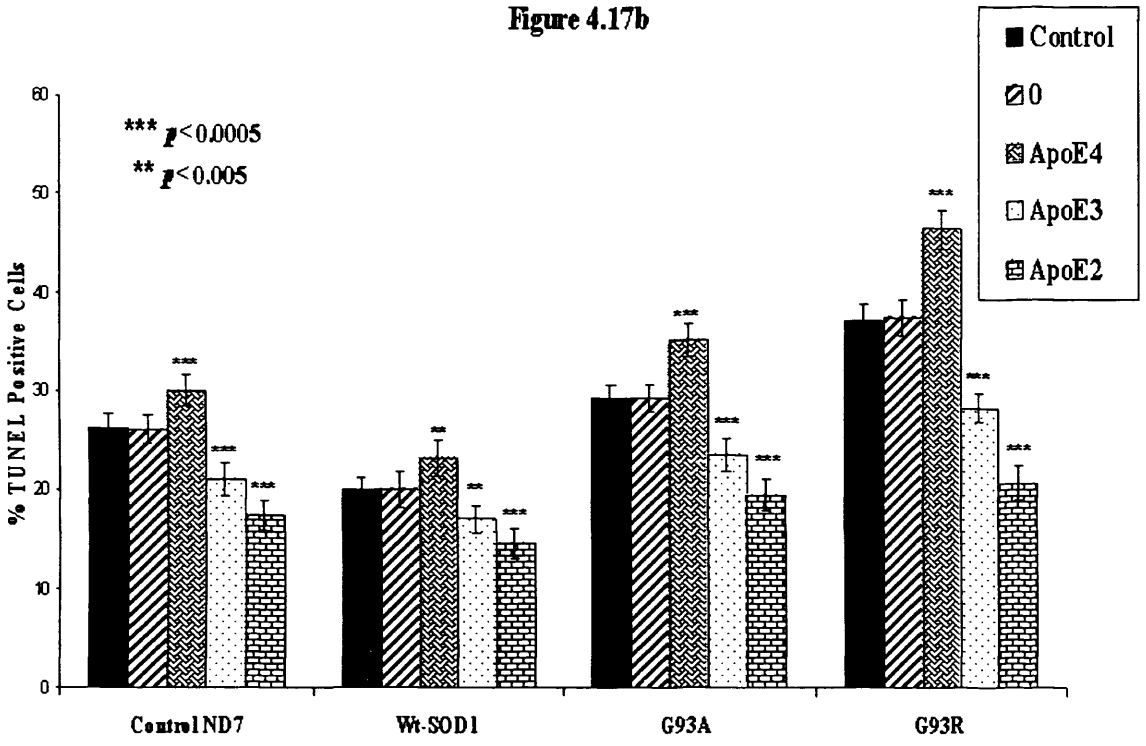



**Figure 4.17 Effect of ApoE isoforms on SOD1 stable cells following serum removal plus 1μM *all-trans* retinoic acid**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were incubated with ApoE protein 1h prior to treatment of serum deprivation plus 1μM *all-trans* retinoic acid for 24h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=3) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with the respective control-vector, wt and G93A and G93R-SOD1 mutant control cells.

### 4.7.3 IFN-γ administration

To further investigate the effects of ApoE alleles on the cells the cells were subjected to a range of stresses, which had been investigated previously. On exposure to IFN-γ the G93A and G93R mutant cells reacted just as with previous experiments. On addition of ApoE4 the G93A and G93R mutants both exhibited an increase in total cell death with death increasing by approximately 29% and 32% respectively compared to respective control ($p<0.0005$); (Figure 4.18a). On addition of ApoE3 the mutants G93A and G93R exhibited a reduction in death with death being reduced by approximately 10% ($p<0.01$) and 22% ($p<0.0005$) respectively compared to respective control (Figure 4.18a). The greatest reduction in death was observed on addition of ApoE2 with death being reduced by approximately 26% and 44% for G93A and G93R respectively compared to respective control ($p<0.0005$). Wt-SOD1 over-expressing cells exhibited a similar trend with addition of ApoE4 resulting in an increase in total cell death of approximately 17% compared to control ($p<0.005$). On addition of ApoE3 and ApoE2 wt-SOD1 cells exhibited a decrease in cell death with death being reduced by approximately 9% and 18% ($p<0.005$) respectively compared to control.

A similar trend was observed when death was analysed using TUNEL analysis. The number TUNEL positive cells increased across all cells on addition of ApoE4. The G93A and G93R mutants exhibited an increase in the number of TUNEL positive cells by approximately 35% and 36% respectively on addition of ApoE4 compared to respective control ($p<0.0005$); (Figure 4.18b). On addition of ApoE4 to wt-SOD1 cells a similar increase in the number of TUNEL positive cells was observed with number of TUNEL positive cells increasing by approximately 22% compared to control ($p<0.0005$); (Figure 4.18b). ApoE3 and ApoE2 addition resulted in a decrease in the number of TUNEL positive cells with G93A cells exhibiting a reduction of approximately 16% and 25% respectively compared to control ($p<0.0005$). G93R cells exhibited a similar trend on addition of ApoE3 and ApoE2 with the number of TUNEL positive cells decreasing by approximately 22% and 43% respectively compared to control ($p<0.0005$). On addition of ApoE3 and ApoE2 to wt-SOD1 cells the number of TUNEL positive cells decreased by approximately 14% ($p<0.05$) and 25% ($p<0.0005$) respectively compared to control.

**Figure 4.18a**

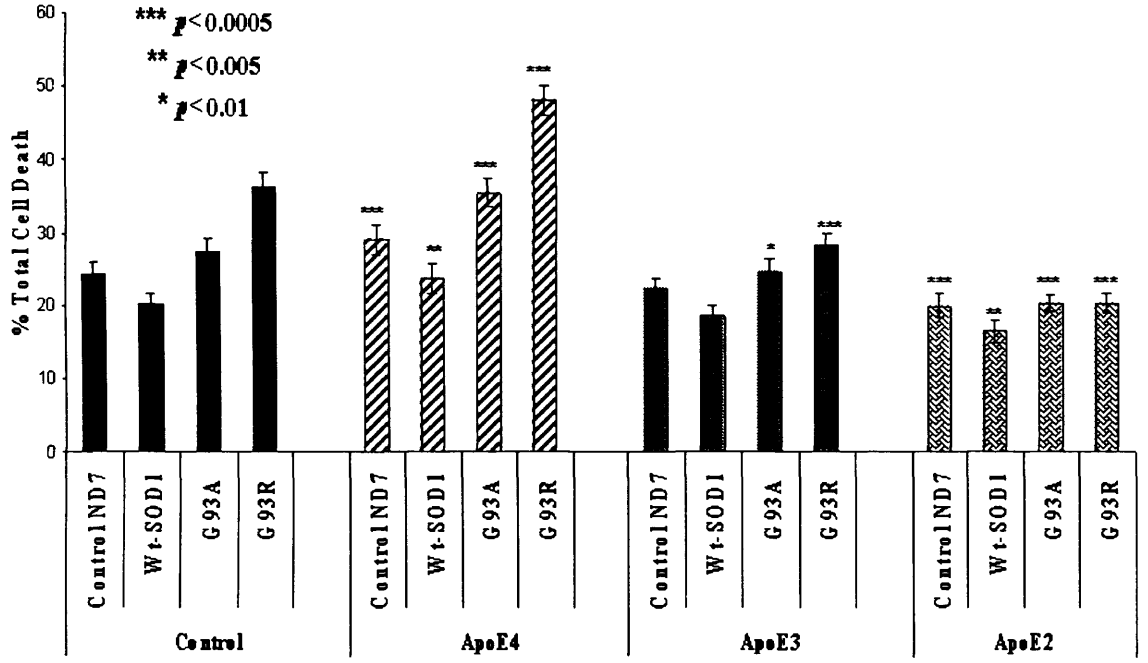



**Figure 4.18 Effect of ApoE isoforms on SOD1 stable cells following IFN-γ administration**

**a.** ND7 cell death following 24h of 50ng/ml IFN-γ or 50ng/ml IFN-γ after incubation with 50ng/ml Apolipoprotein-E (ApoE), ApoE4, ApoE3 or ApoE2. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of IFN-γ treatment of engineered ND7 cells expressing wt or mutant SOD1. Cells were incubated with ApoE isoforms 1h prior to stress. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=5. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p<0.0005$). *, **, *** indicates statistically significant difference between means, when comparing with the respective control-vector, wt and G93A and G93R-SOD1 mutant control cells (cells treated with control solution obtained from control cells).

**Figure 4.18b**

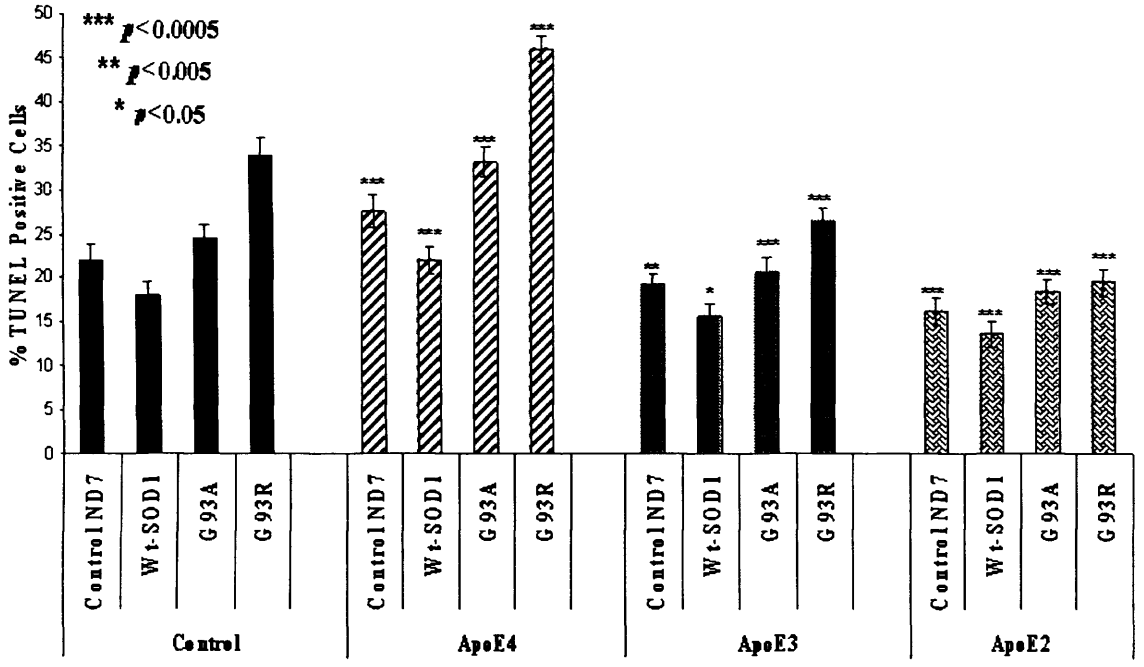



**Figure 4.18 Effect of ApoE isoforms on SOD1 stable cells following IFN-γ administration**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were incubated with ApoE protein 1h prior to treatment of 50ng/ml IFN-γ for 24h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=3) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with the respective control-vector, wt and G93A and G93R-SOD1 mutant control cells.

### 4.7.4 Staurosporine treatment

On addition of ApoE4 mutant cell death increased for both mutants. G93A mutant cell death increased on addition of ApoE4 by approximately 34%, 25% and 24% at 2h, 4h and 6h respectively compared to respective control ($p<0.0005$) (Figure 4.19a). G93R mutant cells exhibited an increase in total cell death of approximately 43%, 32% and 31% at 2h, 4h and 6h respectively on addition of ApoE4 compared to respective control ($p<0.0005$); (Figure 4.19a). A similar increase in total cell death was observed in wt-SOD1 cells with death increasing by approximately 26%, 23% and 30% at 2h, 4h and 6hr respectively on addition of ApoE4 compared to respective control ($p<0.0005$). ApoE3 addition resulted in a decrease in total cell death of all cells. G93A mutant cell exhibited a decrease of approximately 19%, 20% and 19% at 2h, 4h and 6h respectively on addition of ApoE3 compared to respective control ($p<0.0005$). On addition of ApoE3, G93R cells exhibited a decrease in cell death of approximately 26%, 21% and 16% at 2h, 4h ad 6h respectively compared to respective control ($p<0.0005$). Wt-SOD1 cells exhibited a decrease in cell death of approximately 22%, 20% and 22% at 2h, 4h and 6h respectively on addition of ApoE3 compared to respective control ($p<0.0005$). Total cell death was further reduced by addition of ApoE2. G93A mutant cells exhibited a decrease in total cell death of approximately 59%, 57% and 50% at 2h, 4h and 6h respectively on addition of ApoE2 compared to respective control ($p<0.0005$). G93R mutant cell death was reduced by approximately 59%, 56% and 53% at 2h, 4h and 6h respectively on addition of ApoE2 compared to respective control ($p<0.0005$). Wt-SOD1 cells also exhibited a reduction in cell death on addition of ApoE2 with death being reduced by approximately 47%, 43% and 39% at 2h, 4h and 6h respectively compared to respective control ($p<0.0005$).

TUNEL analysis revealed similar results with ApoE4 bringing about approximately a 39%, 27% and 36% increase in the number of TUNEL positive cells at 2h, 4h and 6h respectively for G93A cells compared to control ($p<0.0005$); (Figure 4.19b). G93R cells exhibited a similar increase in the number of TUNEL positive cells with numbers increasing by approximately 46%, 35% and 31% at 2h, 4h and 6h respectively compared to control ($p<0.0005$); (Figure 4.19b). ApoE3 addition resulted in a reduction in the number of TUNEL positive cells with number decreasing by approximately 20%, 20% and 17% for

G93A and 27%, 21% and 17% for G93R at 2h, 4h, and 6h respectively compared to respective control ($p<0.0005$). Addition of ApoE2 reduced the number of TUNEL positive cells by a larger extent with number reduced by approximately 63%, 58% and 51% for G93A and 63%, 57% and 56% for G93R cells at 2h, 4h and 6h respectively compared to respective control ($p<0.0005$). In wt-SOD1 cells addition of ApoE4 increased the number of TUNEL positive cells by approximately 31%, 24% and 35% at 2h, 4h, and 6h respectively compared to control ($p<0.0005$). ApoE3 addition to wt-SOD1 cells reduced the number of TUNEL positive cells by approximately 21% ($p<0.05$), 22% ($p<0.0005$) and 20% ($p<0.0005$) at 2h, 4h and 6h respectively compared to control. ApoE2 addition to wt-SOD1 reduced the number of TUNEL positive cells by approximately 47%, 48% and 40% at 2h, 4h and 6h respectively compared to control ($p<0.0005$).

**Figure 4.19a**

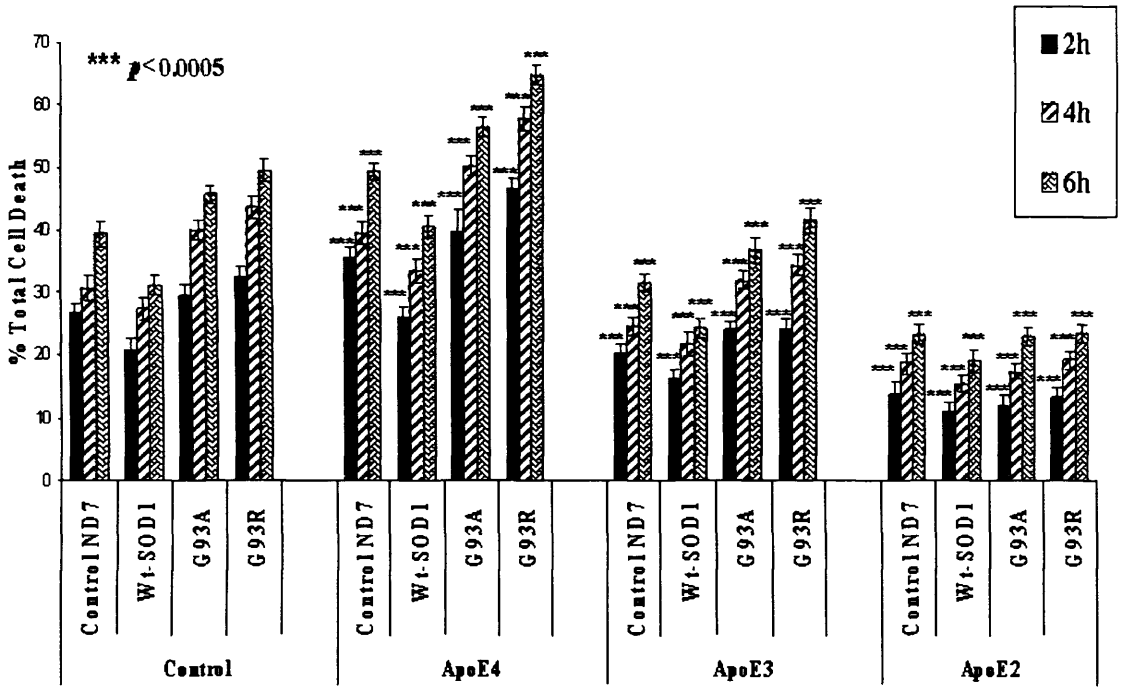



**Figure 4.19 Effect of ApoE isoforms on SOD1 stable cells following staurosporine treatment**

**a.** ND7 cell death following 24h of 1μM staurosporine or 1μM staurosporine after incubation with 50ng/ml Apolipoprotein-E (ApoE), ApoE4, ApoE3 or ApoE2. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of staurosporine treatment of engineered ND7 cells expressing wt or mutant SOD1. Cells were incubated with ApoE isoforms 1h prior to stress. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=5. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p$<0.0005). *, **, *** indicates statistically significant difference between means, when comparing with the respective control-vector, wt and G93A and G93R-SOD1 mutant control cells (cells treated with control solution obtained from control cells).

Figure 4.19b

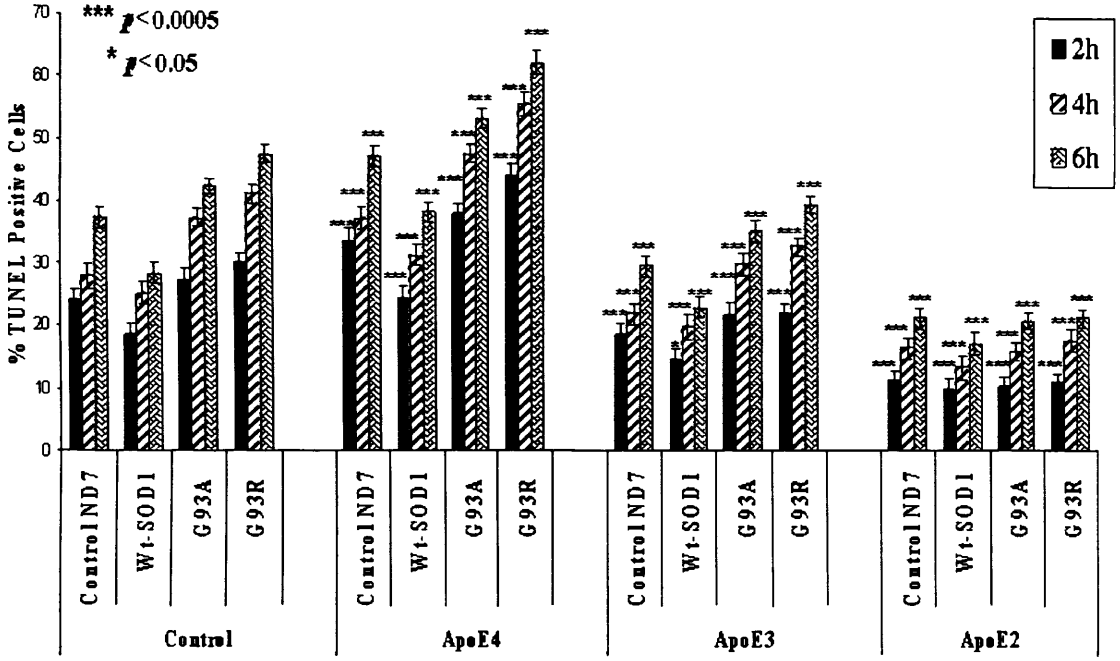



**Figure 4.19 Effect of ApoE isoforms on SOD1 stable cells following staurosporine treatment**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were incubated with ApoE protein 1h prior to treatment of 1μM staurosporine for 24h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=3) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with the respective control-vector, wt and G93A and G93R-SOD1 mutant control cells.

### 4.7.5 Camptothecin administration

On exposure to camptothecin and addition of ApoE4 to G93A and G93R mutant cells total cell death increased by approximately 30% and 33% respectively compared to respective control ($p<0.0005$); (Figure 4.20a). ApoE3 addition, on the other hand, corresponded to a reduction in cell death with G93A and G93R mutants exhibiting a reduction of approximately 12% ($p<0.005$) and 19% ($p<0.0005$) respectively compared to respective control (Figure 4.20a). A greater reduction was observed on addition of ApoE2 with cell death decreasing by approximately 22% and 33% respectively compared to respective control ($p<0.0005$). As with previous experiments wt-SOD1 expressing cells exhibited a similar trend to the mutants with ApoE4 corresponding to an increase in cell death with death rising by approximately 23% compared to control ($p<0.0005$). Addition of ApoE3 and ApoE2 resulted in a reduction in cell death with death being reduced by approximately 5% and 17% ($p<0.005$) respectively compared to control.

TUNEL analysis revealed an increase in the number of TUNEL positive cells on addition of ApoE4 as with previous experiments. Addition of ApoE4 increased the number of TUNEL positive cells by approximately 31% and 35% for G93A and G93R respectively compared to respective control ($p<0.0005$); (Figure 4.20b). ApoE3 addition resulted in a reduction in the number of TUNEL positive cells with the number being reduced by approximately 18% for both G93A and G93R mutant cells compared to respective control ($p<0.0005$); (Figure 4.20b). ApoE2 addition lead to a further reduction in the number of TUNEL positive cells with G93A and G93R exhibiting a reduction of approximately 35% and 40% respectively compared to respective control ($p<0.0005$). Wt-SOD1 cells exhibited an increase in the number of TUNEL positive cells on addition of ApoE4 with the number of TUNEL positive cells increasing by approximately 21% compared to control ($p<0.005$). Addition of ApoE3 and ApoE2 reduced the number of TUNEL positive wt-SOD1 cells with number of TUNEL positive cells decreasing by approximately 13% and 28% ($p<0.0005$) respectively compared to control.

**Figure 4.20a**

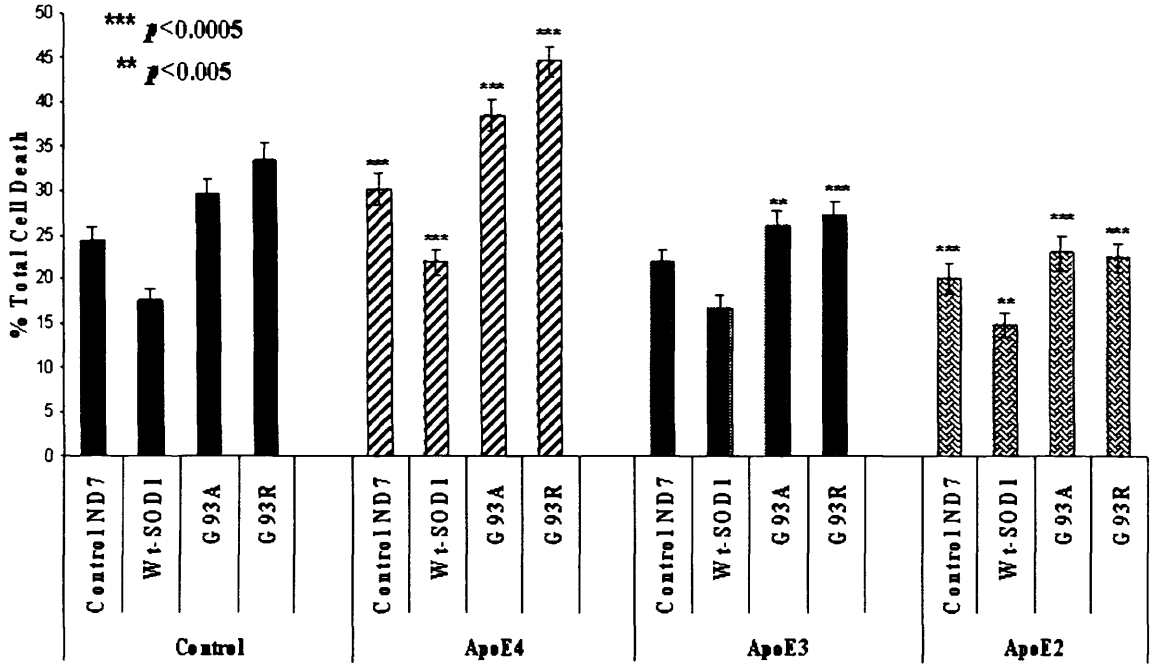



**Figure 4.20 Effect of ApoE isoforms on SOD1 stable cells following camptothecin administration**

**a.** ND7 cell death following 24h of 1μM camptothecin or 1μM camptothecin after incubation with 50/ng/ml Apolipoprotein-E (ApoE) alleles, ApoE4, ApoE3 or ApoE2. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of camptothecin treatment of engineered ND7 cells expressing wt or mutant SOD1. Cells were incubated with ApoE isoforms 1h prior to stress. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=5. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p<0.0005$). *, **, *** indicates statistically significant difference between means, when comparing with the respective control-vector, wt and G93A and G93R-SOD1 mutant control cells (cells treated with control solution obtained from control cells).

**Figure 4.20b**

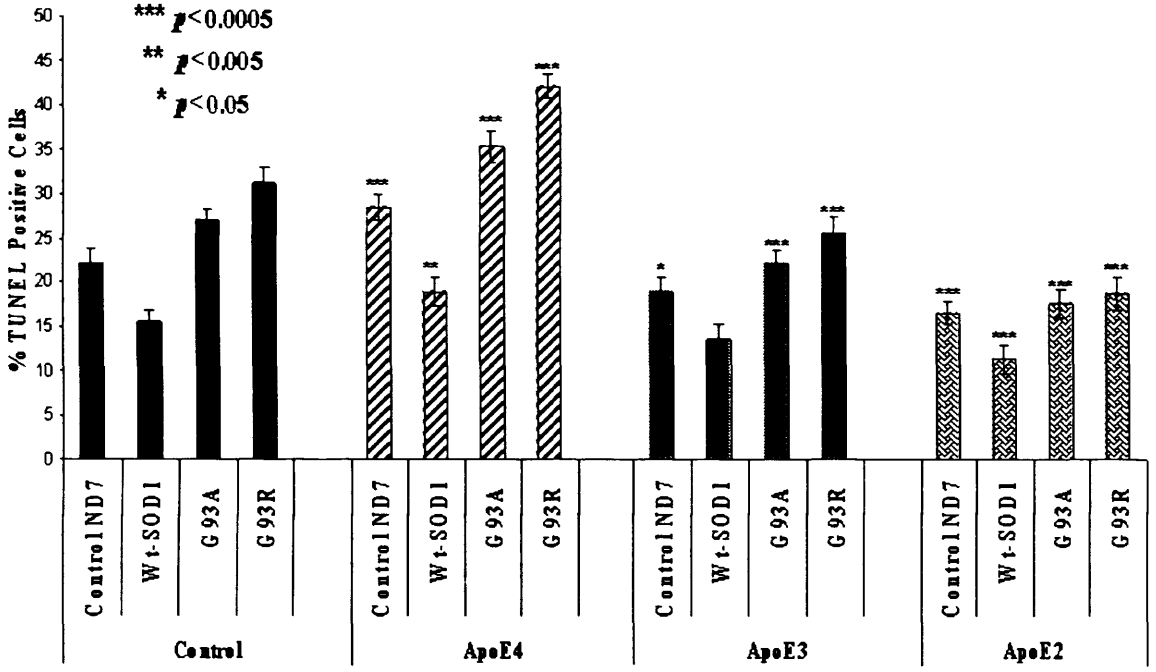



**Figure 4.20 Effect of ApoE isoforms on SOD1 stable cells following camptothecin administration**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were incubated with ApoE protein 1h prior to treatment of 1μM camptothecin for 24h. Bars represent mean $^{\pm}$ S.D. calculated for triplicate counts per sample (n=3) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with the respective control-vector, wt and G93A and G93R-SOD1 mutant control cells.

## 4.8 Discussion

This chapter describes the protective effect of heat shock proteins in the *in vitro* model system of mutant-SOD1-associated toxicity that was described in Chapter 3. HSV-based viral vectors were utilised in order to efficiently express Hsp27 or Hsp70. In summary, the wt-SOD1, mutant SOD1 and empty vector control expressing cell lines were infected with the viral vectors or the relevant control viruses and subsequently subjected to various stresses as in the experiments described in Chapter 3, including serum withdrawal, serum withdrawal plus 1μM retinoic acid, IFN-γ, staurosporine, and camptothecin. The response of the cell lines to the above death inducing stimuli was quantified by different methods of cell death assessment, such as trypan blue exclusion to establish total cell death and TUNEL analysis to establish the number of apoptotic cells.

Briefly, the results indicate that the exogenous expression of Hsp27 and Hsp70 in an established *in vitro* mammalian model over-expressing G93A and G93R SOD1 mutants, confer protection against a wide range of lethal stimuli. Wt-SOD1 cells exhibited increased levels of death with Hsp70 infection bringing about a larger increase in death than Hsp27. Interestingly Hsp70 was much more effective in rescuing the G93R mutant than Hsp27, with the opposite being true for the G93A mutant. Furthermore, a joint infection of Hsp27 and Hsp70 rescued both G93A and G93R SOD1 mutants more effectively than single viral infections with Hsp27 or Hsp70 alone. In addition the enhanced cell death observed in the wt-SOD1 cells on infection of Hsp27 or Hsp70 alone was abolished on expression of the combination of Hsp27 and Hsp70 suggesting that Hsp27 and Hsp70 may act in synergy.

The G93R mutation is much more severe than the G93A mutation and this can be seen throughout all the stresses tested and parallels the much more severe disease phenotype and early onset observed in ALS patients with the G93R mutation. The levels of death obtained with G93A mutation were not as high as the G93R mutation and only slightly higher than control levels. However, the protective effect observed with Hsp27 or Hsp70 reduced the death observed in the G93A cells below that observed in control cells treated with these Hsps. Here the Hsps probably provide more than a general cytoprotective effect and impose their protective effect on the SOD1 mutant. This protective effect is probably

through restoration of normal protein conformation rather than degradation of misfolded protein, as the addition of Hsps lowers the levels of death in both the mutants by a greater amount than that observed in control cells. The reduction in death of the mutants, beyond the levels of death observed in the control cells would be expected if the mutant was refolded to obtain wild-type conformation and thereby able to exert its wild-type anti-apoptotic properties through over-expression of wt-SOD1 protein.

A possible explanation for the increased cell death observed in the wt-SOD1 over-expressing cell line following Hsp27 or Hsp70 infection may be the increased expression of basal levels of SOD1. It is known that over-expression of Hsps results in the corresponding up-regulation of wt-SOD1 (Hass et al., 1988; Loven et al., 1985; Yoo et al., 1999); and would lead to an increase in cell death through an increase in oxidative stress. It is known that over-expression of wt-SOD1 above and beyond a certain threshold can be deleterious and harmful to the cell (Omar et al., 1990) as the wt-SOD1 catalyses the reaction of $O^{2-}$ with NO resulting in the generation of $ONOO^-$ which subsequently leads to generation of oxidative stress. The Hsp27 and Hsp70 combination in the wt-SOD1 cells brought levels of cell death down towards control levels. This maybe brought about by the combination of Hsp27 and Hsp70 activating a proteolytic pathway and degrading any excess wt-SOD1 or the combination self-regulating each other and preventing over-excessive production of wt-SOD1. Alternatively, the two factors together may have a sufficiently potent protective effect to neutralise the damage brought about by a large excess of wt-SOD1.

The increased susceptibility of the G93A and G93R SOD1 mutants to all stresses tested is greatly reduced by caspase inhibitors or by both single infections of Hsp27 and Hsp70 and by a greater amount by dual infections of both Hsp27 and Hsp70. In an attempt to identify a mechanism of protection by Hsp27 or Hsp70, the serum withdrawal protocol was chosen initially to test whether the use of specific caspase inhibitors and/or Hsp27 or Hsp70 or Hsp27 and Hsp70 over-expression could reduce the levels of cell death, particularly in the G93A and G93R-SOD1 mutant expressing cells. The results presented in section 4.3 indicate that following serum withdrawal the mutants G93A and G93R increase toxicity in ND7 cells by increasing the activation of components of the caspase cascade in these cells, resulting in an increase in cell death. This sensitivity was abolished by inhibiting

either caspase-8 or caspase-9 or by over-expressing Hsp27, Hsp70 or Hsp27 and Hsp70 combination. Interestingly, although the G93A or G93R mutant expressing cells were markedly protected by either caspase inhibitors or by Hsp27, Hsp70 or Hsp27 and Hsp70 overexpression, there was no additive effect between the Hsps and the caspase inhibitors for further protection to occur.

This suggests that the majority of the enhanced cell death seen upon over-expression of G93A or G93R SOD1 mutations is apoptotic and propagated through caspase dependent mechanisms, which can be blocked by either caspase inhibitors or by Hsp27 and/or Hsp70. The increase in cell death on treatment with zVAD-fmk and addition of either Hsp27 or Hsp70 may be put down to an unfavourable interaction between the inhibitor and the Hsp27 or Hsp70 rather than toxicity of the virus itself, simply because if this was the case one would expect a corresponding increase on treatment of GFP+zVAD. The increase in death observed on treatment with zVAD-fmk and Hsp27 or Hsp70 compared to zVAD-fmk alone could be as a result of a competition between zVAD-fmk and Hsp27 or Hsp70 for the same molecules in the apoptotic cascade resulting in hindrance of the anti-apoptotic properties of both and leading to an increase in death when compared to treatment of zVAD-fmk alone.

Previously, Wagstaff et al. (1999) demonstrated that neuronal cells can be protected against harmful stimuli inducing apoptosis by Hsp27, but the exact mechanism of protection against these stimuli as yet still remains unclear. Recently, Zourlidou et al. (2004) demonstrated the protective effects of Hsp27 against the damaging effects of over-expressing wild-type and mutant α-synuclein, in ND7 cells. Furthermore, Zourlidou et al. (2004) found the death brought about by mutant α-synuclein to be apoptotic with caspase inhibitors and Hsp27 blocking all death and a joint treatment of caspase inhibitor and Hsp27 not producing an extra protective effect. However, Zourlidou et al. (2004) found Hsp70 did not provide a strong protective effect against α-synuclein toxicity under stresses tested, whilst simultaneous overexpression of Hsp27 and Hsp70 was as protective as Hsp27 expression alone.

Hence the findings in this chapter could be explained partly by the fact that both Hsp27 and Hsp70 have multiple anti-apoptotic actions (Garrido et al., 2001; Concannon et

al., 2003; Parcellier et al., 2003). The precise mechanism of protection by Hsp27 and/or Hsp70 in various situations is obviously very complex and as yet remains incompletely understood. Over-expression of Hsp27 protects against apoptotic cell death triggered by a range of stimuli, including hyperthermia, oxidative stress, staurosporine, ligation of the Fas/Apo-1/CD95 death receptor, and cytotoxic drugs (Garrido et al., 1996 & 1997; Mehlen et al., 1996). Various distinct mechanisms have been put forward to account for the anti-apoptotic properties of Hsp27 such as its ability to increase the anti-oxidant defence of cells by decreasing reactive oxygen species cell content (Mehlen et al., 1996) and its ability to stabilize actin microfilaments (Lavoie et al., 1995).

In the intrinsic pathway of apoptosis Hsp27 prevents the formation of the apoptosome and thereby the subsequent activation of caspases by directly sequestering cytochrome *c* (Bruey et al., 2000; Garrido et al., 1999). Hsp27 has also been shown to interact with caspase-3 (Concannon et al., 2001; Pandey et al., 2000) and the phosphorylated form of Hsp27 has been shown to directly interact with Daxx to prevent cell death (Charette et al., 2000). Hsp70 has the ability to directly bind Apaf-1, thereby preventing the recruitment of procaspase-9 to the apoptosome (Beere et al., 2000; Mosser et al., 2000; Saleh et al., 2000). In addition, Hsp70 can also bind to AIF and as a result prevent AIF-mediated apoptosis (Creagh et al., 2000; Ravagnan et al., 2001). It is also thought that Hsp70 may also protect the cells from energy deprivation and/or ATP depletion associated with cell death (Wong et al., 1998). Furthermore, Hsp70 has also been shown to avert JNK activation (Meriin et al., 1999; Park et al., 2001).

As mentioned previously, Hsp70 was initially proposed to rescue cells from a later stage of apoptosis than any other known survival enhancing drug or protein with its overexpression shown to inhibit downstream morphological changes that are characteristic of dying cells (Jaattela et al., 1998; Li et al., 2000). It has also been found that elevated levels of Hsp70 could prevent both caspase activation and apoptosis-associated nuclear changes (Buzzard et al., 1998). This may provide a possible explanation for why Hsp70 is better poised to rescue the G93R SOD1 mutant than Hsp27. Under all stresses tested Hsp70 reduced death of G93R SOD1 mutant by a greater amount than was observed with Hsp27. The G93R SOD1 mutation has a much more severe disease phenotype compared to G93A

SOD1 mutation and is associated with a particularly early age of onset and this effect is paralleled throughout all the stresses tested by a greater enhancement of cell death with G93R compared to G93A. It is plausible that as the G93R SOD1 mutant is much more severe, both *in vivo* and in our experiments, that at any one time it maybe further along the apoptotic pathway than the corresponding less severe G93A mutant and therefore Hsp70 would be better placed in rescuing the mutant from death. If this were thought to be true then it would mean that Hsp70 would be able to rescue more severe SOD1 mutants more effectively than Hsp27.

The mechanism by which Hsp27 exerts its protective effects may be through its ability to maintain redox homeostasis and mitochondrial stability. The suggestion that Hsps exert their protective roles at the level of the mitochondrion is not an entirely new concept. Previously Polla et al. (1996) proposed that mitochondria are the targets of the protective effects of Hsps against oxidative stress. Mutant SOD1 has been shown to localise to the mitochondria where it sets off caspase-dependent cell death (Takeuchi et al., 2002). It is possible that mutant SOD1 exerts its effects on mitochondrial SOD2 by relocating to the mitochondria where the SOD1 mutant catalyses the reaction of superoxide radical ($O_2^{\cdot -}$) with nitric oxide (NO) to generate peroxynitrite ($ONOO^-$).

Mn-SOD (SOD2), which is the enzyme responsible for the detoxification of intramitochondrial O2.- radical, has been shown to be nitrated and inactivated both *in vitro* and *in vivo* (MacMillan-Crow et al., 1996; Quijano et al., 2001; Yamakura et al., 1998). Mn-SOD inactivation involves peroxynitrite-mediated nitration of tyrosine-34 (Yamakura et al., 1998) with the subsequent formation of a nitrating species, which cannot only self-nitrate (Tyr-34) but one that also nitrates remote tyrosine residues (Quijano et al., 2001). Mn-SOD inactivation may in turn promote a vicious cycle favouring further peroxynitrite formation and lead to further mitochondrial oxidative damage. Mitochondria possess the ability to detoxify peroxynitrite by one or more scavenging systems with one of these systems being the reaction with glutathione (Castro et al., 1998). Hsp27 can increase intracellular levels of glutathione (Mehlen et al., 1996) and this may be a possible additional mechanism by which Hsp27 exerts its protective effect.

Following caspase inhibitor treatment, the G93A and G93R SOD1 mutants showed a substantial reduction in death. Both caspase-8 and caspase-9 inhibitors reduced the levels of death to that observed with the pan-caspase inhibitor zVAD. This indicates that in this present model system the majority of death is triggered through caspase dependent mechanisms and in particular through caspase-8 and caspase-9. However, on addition of caspase inhibitors and Hsps in combination, no further additive protection was observed. Therefore, the anti-apoptotic actions of Hsp27 and Hsp70 in inhibiting caspases are clearly very important in this present system but one cannot exclude the fact there could be an involvement of the chaperone activity of Hsp27 or Hsp70, which might assist in the folding of potentially aggregated mutant SOD1 or that the Hsps may play a role in protein degradation, and set about degrading the toxic SOD1 mutant. Shimura et al. (2004) have shown that Hsp27 may function in protein degradation in an ubiquitin-independent manner while Parcellier et al. (2003a) have shown that under conditions of stress Hsp27 favours ubiquitinated and phosphorylated I-κBα (main inhibitor of NF-κB) proteosomal degradation. The latter finding suggests a novel function for Hsp27 that provides another explanation for its antiapoptotic activities through the increase in NF-κB activity.

Addition of ApoE provided some interesting results. Addition of ApoE2 or ApoE3 appeared to provide a protective effect in all SOD1 cells on exposure to a range of stresses whereas ApoE4 increased the levels of death in these cells. ApoE2 provided the greatest level of protection by reducing death by approximately 18% and 33% for G93A and G93R respectively compared to control ($p<0.0005$) on exposure to conditions of serum withdrawal. The protective effect of ApoE2 and deleterious effect of ApoE4 was reflected across all the cells under a wide-range of stresses tested.

Among patients with MND (motor neuron disease), 2 (Al-Chalabi et al., 1996; Smith et al., 1996) of 4 published studies exhibit trends toward APOE4 alleles being associated with faster progression of disease. The only study examining APOE2 and MND progression demonstrates that patients with MND and APOE2 alleles live significantly longer than those individuals without this allele (Moulard et al., 1996). Each of these studies is open to methodological criticisms however their results are consistent with one another and with the data on disease in the CNS (central nervous system): APOE4 is associated with a worse

prognosis and APOE2 with a better prognosis for a given neurological disease. As yet, associations between APOE allele type and disease progression have not been explored in the ALS/parkinsonism/dementia complex of Guam, in IBM (inclusion body myositis), in amyloidic neuropathy, or in any other PNS (peripheral nervous system) disease.

APOE could influence the progression of these very different CNS and PNS diseases through a variety of different mechanisms. One wide-ranging hypothesis is that certain APOE alleles confer an altered resistance to disease. Evidence from *in vitro* studies suggests different isoforms of ApoE confer different susceptibilities to oxidative stress (Miyata et al., 1996). These differences may be mediated to some extent by the neuron's ability to use growth factors; indeed, *in vitro* ApoE is known to bind and potentiate the survival-promoting activity of ciliary neurotrophic factor (Gutman et al., 1997). In addition, the size and shape of axons from APOE-knockout mice (Fullerton et al., 1998) are morphologically similar to axons from rats treated with nerve growth factor antiserums (Gold et al., 1991). An alternative broad hypothesis is that certain ApoE isoforms confer an enhanced regenerative response to disease. For instance, in a variety of cell lines, ApoE3 is associated with increased neurite outgrowth, while ApoE4 is associated with decreased neurite outgrowth (Nathan et al., 1994; Bellosta et al., 1995; Holtzman et al., 1995). This altered regeneration may yet again be caused by an allele-specific use of growth factors. On the other hand, the response may be dependent on isoform-specific alterations in neuronal adhesion (Huang et al., 1995) cytoskeletal stability (Gutman et al., 1997), or the use of lipids for the expanding cell membrane.

Alternatively, it is thought that the protective effect of ApoE2 over ApoE4 may be down to the ability of ApoE2 to bind HNE (4-hydroxynonenal) to a greater extent than ApoE4 and as such protect against apoptosis induced by HNE (Pedersen et al., 2000). The differential ability of the ApoE isoforms to protect against HNE can be put down to the number of cysteine residues possessed by each form with ApoE2 possessing two, ApoE3 having one and ApoE4 none (Pederson et al., 2000). A higher concentration of free HNE in the cerebrospinal fluid of ALS patients relative to controls (Smith et al., 1998) and an increase protein modification by HNE in ALS spinal cord relative to control spinal cord (Pedersen et al., 1998) have been reported. The latter findings are of particular interest as

one of the proteins modified by HNE in ALS spinal cord appears to be EAAT2, and HNE has been shown to impair glutamate transport in NSC-19 cells (Pedersen et al., 1999). It has also been reported than HNE impairs the function of ion-motive ATPases, glucose and glutamate transporters in primary neuronal systems (Keller et al., 1997; Mark et al., 1997) and glutamate transporters in astrocytes (Blanc et al., 1998), indicating that HNE promotes excitotoxic damage. In addition it is thought the Par-4 may be a mediator of HNE-induced motor neuron death initiated by oxyradical attack on membrane lipids (Pedersen et al., 2000). All this evidence and the results of this study suggest that APOE alleles influence the progression of ALS; APOE4 confers an increased risk of disease and a worse prognosis, while the presence of APOE2 confers a decreased risk of disease and a better prognosis. Further studies are required to solidify this relationship.

Although the cellular model presented here is not the ideal cellular model to study ALS - it remains a very useful mammalian neuronal system where an interesting effect of wt-SOD1 and the FALS-associated mutants has been observed (Chapter 3). This system has an obvious advantage over the reported use of various non-neuronal or non-mammalian systems. It has allowed the testing for the first time of the protective effect of various Hsps both singly and in combination, against the mutant SOD1-induced cell death in a mammalian neuronal system. Most importantly, this system helped to conclude that Hsp27, Hsp70 and to a greater extent the Hsp27 and Hsp70 combination is a potent neuroprotective agent against mutant SOD1-associated toxicity; this is the first report to show alleviation of mutant SOD1-neurotoxicity in an *in vitro* mammalian neuronal system by Hsp27 and by the combination of Hsp27 and Hsp70 (Patel et al., 2005).

The distinct death stimuli used at various time intervals (serum withdrawal, serum withdrawal plus 1μM retinoic acid, IFN-γ, staurosporine and camptothecin) were selected on the basis of having been previously characterised in ND7 cells (as explained in Chapter 3) and being representative stresses inducing both apoptotic and necrotic cell death in neuronal cells, which are modes of neuronal death implicated in FALS pathology (see review by Guégan et al., 2003). In addition, there is accumulating evidence that protein misfolding as well as proteasome system activation take place during apoptosis (Soldatenkov et al., 1998). Therefore, the apoptotic stimuli used here represent some of the conditions that are

encountered in FALS, such as protein misfolding and proteasome system activation, which are known to occur during the course of apoptosis (Guégan et al., 2003). In addition, the highly efficient gene delivery of Hsps by the HSV viral vector system assists in the study of the protective effects of Hsps and also enables the biochemical dissection of the mechanism of protection in this system.

The cell death assessment by trypan blue exclusion assay was more time consuming than the other death assessment as a result of the larger sample numbers that had to be counted in a haemocytometer. This means that the samples had to be left on ice for a relatively longer time period and, therefore, it is possible that as apoptotic cells lose their membrane integrity relatively faster they uptake the trypan blue dye, resulting in a high number of dead or dying blue cells in the relevant experiments. On the other hand TUNEL was performed on cells adhered to glass slides and hence after treatment the media was aspired, the cells washed, fixed and permeabilized prior to TUNEL. As a result the cells that had died and as a result detached from the plate at the end of the treatment were not counted. However, by comparing the percentage of TUNEL positive cells per cell line a fairly accurate estimate of the relative levels of apoptotic cell death in these different cell lines can be obtained. It is usually suggested in the literature that different methods of cell death assessment as well as assessment of cell death at multiple time points should be applied before one concludes on certain research questions.

From the present study it becomes apparent that no single method of cell death quantification is precise and totally informative and that any conclusions drawn should be done so very carefully after reconfirming a trend in cell death susceptibility by an alternative method. Trypan blue is thought to have the disadvantage of colour intensity variation (amount of dye uptake varies depending on cell state) which can result in an under or over-estimation in the number of dead cells. However this can be overcome by setting the same thresholds for all experiments, in which cell counts are taken, in a blind fashion by the same researcher. On the whole, both trypan blue and TUNEL assay have been used extensively and remain valid and reliable assays for cell viability and apoptotic cell death assessment respectively. By comparing the cell death of a cell line to the appropriate control cell line, an

effect due to treatment can be assessed by comparing the relative differences, as is the case in the present study.

Finally, some other points that need to be taken into account include the fact that the SOD1 cDNA is human and the disease mutations are found in humans with autosomal dominant FALS, whereas the ND7 cell line is a rat/mouse hybrid, with the Hsp27 being from Chinese hamster and Hsp70 being the human inducible Hsp70. However, Hsps are highly conserved proteins and trans-species experiments are performed regularly and often successfully. It is difficult to know whether or not the species origin of the cDNAs, used in this present study, is of significance but one needs to take them into consideration. However in this study the Hsps were effective in reducing the mutant associated toxicity and were protective for the mutants under all stresses tested.

Further support for the findings presented in this chapter could be obtained from experiments on the aggregation properties of wt and mutant SOD1. Experiments could be conducted to ascertain whether aggregates of wt-SOD1 or its mutants form within the cells and whether formation of aggregates corresponds to a decrease in cell survival; and in addition, whether addition of Hsp's reduces the mean number of aggregates present in the cell and thereby increase survival. These experiments would provide a better understanding of the protection conferred by the Hsp's and may provide an explanation as to how single infections of Hsp increase death in wt-SOD1 cells and the dual combination reduce it.

It is very likely that multiple pathways are involved in the protection conferred by Hsp27 and/or Hsp70, and it will be necessary to investigate the contribution of these other pathways to the effects reported here. It may be useful to perform caspase activation assays, in the model presented here, to investigate if caspase processing varies in response to different stresses in the presence or absence of Hsp27 and/or Hsp70 over expression, and to extend these investigations by transfecting dominant negative caspase constructs into the various SOD1 cell lines. Alternatively, one may use naturally occurring caspase inhibitor genes such as caspase-9S or FLIP. A similar approach could be utilized to investigate other signal transduction pathways involved in cell survival for instance those involving Akt and p42/p44 MAP kinase.

In summary, this work has demonstrated for the first time that mammalian neuronal cells over-expressing FALS-associated SOD1 disease mutants G93A and G93R are protected from various death inducing stimuli by exogenous Hsp27 and Hsp70, and moreover, to a greater extent, by a combination of both. Wt-SOD1 over-expressing cells were not protected by delivery of the single exogenous Hsps but death was reduced back down toward control GFP levels by dual infection of Hsp27 and Hsp70. Both Hsp27 and Hsp70 seem to exert their protective effects by interfering with the caspase-cascade. Further studies are required to clarify the mechanisms of neuroprotection offered by Hsps, as there appears to be growing evidence of the therapeutic benefit of Hsps in many neurodegenerative diseases. The manipulation of endogenous cellular defence mechanisms such as the heat shock response, by means of nutritional antioxidants or pharmacological compounds, may provide an approach of therapeutic intervention in neurodegeneration. The following chapter further investigates the protective properties of wt-SOD1 and those of Hsp27, Hsp70 and the combination of Hsp27 and Hsp70 in relation to mutant-SOD1 toxicity in a primary cell culture model.

# CHAPTER 5

## Further investigation of the protective effects of Heat Shock Proteins against mutant-SOD1 toxicity in primary cultures of DRGs from Tg-Hsp27 and Tg-Hsp70 mice

## 5.1 Introduction

The previous chapter dealt with the characterisation of the protective effect of heat shock proteins in an *in vitro* model of mutant-SOD1-induced toxicity along with an attempt to identify a possible mechanism by which Hsp27 or Hsp70 conferred protection. This chapter provides further support to the protective effects of Hsp27, Hsp70 and the Hsp27 and Hsp70 combination against mutant-SOD1 toxicity in two further primary cell culture model systems.

The data presented in the previous chapters suggest that, at least in the cellular model system described in this thesis, apoptosis appears to play a primary role in the cellular loss triggered by the wide-range of FALS-relevant stimuli investigated. It was therefore hypothesised that Hsp27 or Hsp70 may confer protection through their anti-apoptotic properties (reviewed in Chapter 1 and Chapter 4) and hence, through use of caspase inhibitors the anti-apoptotic properties were investigated.

Currently chaperones are being considered for the potential treatment of diseases involving protein aggregation and misfolding, from neurodegenerative diseases (Bonini, 2002) to cancer (Scott and Frydman, 2003). The cell has a complex system for maintaining correct protein folding, which begins with the facilitation of folding of nascent proteins, monitoring for the presence of unfolded proteins in various intracellular compartments, and targeting of misfolded or abnormal proteins for degradation. In addition, many facets of protein-protein interactions are also specifically regulated by chaperones. The accumulation of unfolded proteins in the endoplasmic reticulum lumen can elicit the unfolded protein response, which is implicated in the shutdown of protein synthesis, a hallmark of the response to a range of severe cellular stresses (Paschen, 2003). Therefore regulation of the state of protein folding and protein association is a central aspect of normal cellular homeostasis.

Recent work has highlighted the ability of Hsp70 to suppress multiple types of cell death such as necrotic cell death, classical apoptosis, and other programmed cell death pathways that are independent of caspases and not blocked by Bcl-2 (Beere et al., 2000; Jaattela et al., 1998; Nylandsted et al, 2000; Ravagnan et al., 2001; Saleh et al., 2000). Since both apoptotic and

necrotic cell death are involved in FALS, the ability of Hsp70 to reduce both types of cell death makes it an appealing candidate for protection against this neurodegenerative disorder. Understanding the aspects of Hsp70 function that are necessary for protection will identify pathological processes that contribute to cell death and permit future work to target identified pathological mechanisms for neuroprotection.

Unfolded or misfolded proteins have exposed hydrophobic segments that make them more susceptible to aggregation. Protein aggregates are believed to be toxic to the cell (Taylor et al., 2002), therefore to avoid aggregation, abnormal proteins are either kept soluble by molecular chaperones or quickly degraded by the ubiquitin/proteasome system (Hershko and Ciechanover, 1998). Under pathological conditions, the level of abnormal proteins may overcome the ability of the cell to sustain them in a soluble form or degrade them, thereby allowing aggregation to proceed (Cohen, 1999; Zoghbi and Orr, 2000). Protein aggregates can inhibit function of the proteosome, thereby further limiting the cell's ability to dispose of the protein aggregates as well as interfering with the normal processing of certain short-lived proteins (Bence et al., 2001). Protein aggregates have been detected in many chronic neurodegenerative diseases (Kakizuka, 1998; Taylor et al., 2002).

In motoneurons, mitochondrial alterations such as membrane depolarization, decreased activity of respiratory complexes and cytochrome *c* release are prime candidates for involvement in the several aspects of ALS, because of their occurrence at the asymptomatic stage in FALS-SOD1 transgenic mice with relatively slow disease progression (Bendotti et al., 2001; Jung et al., 2002) and in cultured cells expressing the mutant protein (Carrì et al., 1997; Beal et al., 2000). Interestingly, mitochondrial abnormalities have additionally been seen in motor axon terminals of muscle biopsies from patients with early-diagnosed sporadic ALS (Beal et al., 2000; Siklos et al., 1996). Mitochondrial alterations may play a role in the generation of a condition of oxidative stress, and markers of oxidative damage such as increased ROS flux and oxidatively modified proteins, have been found in cultured neuronal cells, and in both transgenic mice and patients with ALS (Carrì et al., 1997; Ferri et al., 2000; Ciriolo et al., 2000; Liu et al., 1999).

Impairments in the respiratory chain, resulting in bioenergetic failure in the motoneurons (Jung et al., 2002; Mattiazzi et al., 2002) and in alterations in the mitochondrial permeability transition pore (MPTP), bringing about leakage of cytochrome c and apoptosis-inducing factor (AIF) (Friedlander, 2003), were suggested to contribute to mitochondria-dependent motoneuron death. This results in the activation of the caspase-cascade leading to programmed cell death, but data supporting an apoptotic form of death of motoneurons in models and patients with ALS are contradictory (Friedlander, 2003; Mighelli et al., 1999). Converging data indicate that the death of cultured cells expressing FALS SOD1 typically proceeds along the mitochondrial apoptotic pathway, through activation of executioner caspases (i.e. caspase-3) (Ghadge et al., 1997; Guégan et al., 2003; Li et al., 2000). However, although components of the programmed cell-death machinery are recruited and interception of the caspase pathway appears to be beneficial to FALS SODl-transgenic mice (Li et al., 2000; Guégan et al., 2003; Inoue et al., 2003), several groups reported that dying neurons in FALS mice exhibit a non-apoptotic morphology (Bendotti et al., 2001; Guégan et al., 2003).

The tendency of misfolded, metal-free SOD1 mutants to form insoluble aggregates in motoneurons is also regarded as an early event in the pathogenesis of ALS (Johnston et al., 2000). Protein aggregation (Lewy-body-like inclusions containing SOD1 and ubiquitin, and an altered neurofilament pattern) has been observed in cultured cells expressing FALS SOD1 (Julien. 2001) and SOD1 is a key component of the neuronal hyaline inclusion (NHI) found in post-mortem tissues of patients with SOD1-associated FALS (Kato et al., 2000). A pathological hallmark that is common to all forms of ALS as well as to animal models is cytoskeletal disorganization in the motoneurons, which is frequently associated with the accumulation of phosphorylated neurofilaments (pNF). Cytoskeletal abnormalities may come about through the alteration of protein phosphorylation by kinases such as p38 MAPK (mitogen activated protein kinase) which is activated in the motoneurons of asymptomatic SOD1-G93A mice (Kato et al., 2000) and in skein-like inclusions of patients with sporadic ALS (Bendotti et al., 2004). Calcineurin (PP2B), a protein phosphatase, is involved in the modulation of tau and is oxidatively inactivated by FALS SOD1 in experimental systems, for instance human cells expressing the mutant SOD1 H46R or G93A as well as in G93A-

SOD1 mice (Ferri et al., 2000). The accumulation of pNF might perturb the axonal transport of substrates that are required for neuronal viability.

In addition, protein aggregates might also impair the activity of proteasomes, as suggested by studies conducted in neuronal cell lines (Allen et al., 2003; Urushitani et al., 2002). In turn, proteasome inhibition could result in further accumulation of aberrant aggregates, which sequester other proteins and lead to the formation of cytoplasmic ubiquitinated inclusion bodies, similar to those found in the SOD1 mutant mice at advanced stages of the disease and in post-mortem tissue from ALS patients (Kato et al., 2000).

It was therefore decided that the present study should be extended to investigate the effects of Hsp27 and Hsp70 in primary cell cultures of DRGs (dorsal root ganglia). The availability of SOD1 viruses (wt-SOD1, G93R-mutant SOD1 and GFP-control) named 1764 27-, constructed in our lab by Dr Yolanda Collaco-Moraes enabled infection and experimentation in primary cultures of DRGs. This virus lacks the ICP27 gene, is deleted for the endogenous LAT P2 regions thereby preventing recombination with the inserted LAT P2-containing expression cassette pR20.5 outside the LAT region. The pR20.5 cassette consists of a central LAT P2 element flanked by two heterologous promoters (CMV-IE and RSV) arranged in a back-to-back orientation, allowing simultaneous expression of either wt-SOD1 or G93R-SOD1 mutant (under the Rous Sarcoma Virus - RSV- promoter) and GFP (under CMV-IE promoter) and enabling long term expression of both transgenes. This cassette was inserted into a plasmid to enable insertional inactivation of the gene encoding virion host shut off protein (vhs), which has been shown to play a role in pathogenesis and latency (Strelow and Leib, 1995) and further disabled the HSV virus. Since ICP27 was deleted, this virus type was grown on an ICP27 complementing cell line (BHK130/2), which over-express ICP27 and thereby complement the defect and allow growth in culture.

The availability of a highly efficient HSV-1-based vector system for delivery of both the Hsp gene and SOD1 or G93R-SOD1-mutant to neuronal cells, in addition to Hsp27 and Hsp70 transgenic mice proved very useful in the present study. As shown in this chapter, it allowed us to further investigate and provide further supporting evidence for the protective

effect of Hsp27, Hsp70 and to a greater extent the Hsp27 and Hsp70 combination against mutant-SOD1 induced toxicity in a primary culture cell model on subjection to a wide-range of FALS relevant stresses.

The aims of this chapter are listed below:
(a) To investigate further the protection conferred by Hsp27, Hsp70 and the combination of Hsp27 and Hsp70 against mutant SOD1 induced toxicity in a primary culture model system

(b) To evaluate the potential of the anti-apoptotic properties of Hsp27, Hsp70 and the Hsp27 and Hsp70 combination in this primary model system and suggest alternative mechanisms of action that should be tested.

## 5.2 Culture of primary neurons

Briefly, DRG (dorsal root ganglia) sensory neuron cultures of the peripheral nervous system were prepared from newborn Harlan Sprague-Dawley rat pups at postnatal day and Hsp27 and Hsp70 transgenic mice (C57 black x) 8-12 weeks old. Sensory neurons were chosen for this study as they are an easy to culture primary neuronal system and it is relatively easy to obtain pure cultures of sensory neurons compared to motor neurons, for which pure cultures are almost impossible to obtain. The animals were sacrificed by cervical dislocation followed by decapitation, ensuring to remove the head above the base of the spine. Using fine scissors cuts were made through the base of the spine and from the start of the neck down on each side of the animal in order to isolate the spinal cord.

Excess tissue was trimmed away from the spine, with care being taken so as not to cut into the spine. The spine was then placed in a fresh dish with the dorsal side (back) facing upwards. The spine was cut along the length to remove the top half and reveal the DRG and spinal cord. The spinal cord was discarded and No.5 forceps used to pinch out the DRGs, which were collected into 900μl HBSS in 35mm Petri dish. Dorsal root ganglia have a sheath that needs to be removed as it hinders dissociation and also introduces excess non-neuronal cells such as glia and fibroblasts into the culture. The dorsal root ganglia (DRG)

cultures of both mice and rats were dissociated in a 100µl mix of 0.3% collegenase/0.1% trypsin for 25 min. Following enzyme treatment 1ml of growth media was added to the dish to stop the enzyme reaction and the ganglia dissociated through either a silanised flame polished pipette or a Gilson blue tip, approximately 10 times with care being taken not to introduce air bubbles as this leads to shearing of the cells.

The dissociated ganglia were then transferred to a 15ml centrifuge tube and spun at 900rpm for 5min at room temperature. To reduce the number of non-neuronals, the cells were spun through a column of 6% metrizamide (Sigma M3383) in culture media following enzyme dissociation. This high-density solution removes the non-neuronals, which are too small to pass through the solution. After centrifugation at 900rpm for 5 minutes, the metrizamide/non-neuronal mixture was tipped off and discarded and the pellet carefully resuspended in appropriate growth media. The cells were plated in 50µl droplets on laminin-coated coverslips. Neurons were left to attach overnight in the incubator at 37°C/5% $CO_2$ and then flooded with appropriate growth media next day. For more detailed methods see section 2.7.2.

## 5.3 Viral Infections of Primary Cells

### 5.3.1 Complementing Cell Lines of Viruses

The stocks of virus used in this study were grown and prepared as described in Chapter 2, section 2.4. The viruses were grown on B130/2 BHK cells to complement the deletions in the disabled viruses and hence enable lytic viral growth (Howard et al., 1998; Thomas et al., 1999). This allows the production of large quantities of the virus, which can be subsequently purified and concentrated into high titre stocks. Each preparation of high titre stock was confirmed to express the transgene, through western analysis of non-complementing ND7 cells infected with the stock.

### 5.3.2 Microscopy of Virally Infected DRGs and Western Blot Analysis

DRGs were infected as described in Chapter 2, section 2.4.2 and then left for 24h to allow overexpression of the transgene prior to treatment. However, GFP was visible earlier than 12h post-infection, and its expression continued to be seen until the end of all treatments. Figure 5.1a shows some DRGs infected with GFP virus. The HSV-vector 17+pR19 has previously been shown to successfully infect ND7 cells and produce large amounts of Hsps (Wagstaff et al., 1999). Figure 5.1b shows the high levels of expression of the transgene after 24h in DRG cells infected with either a control GFP, Hsp27 or Hsp70 expressing virus. The GFP virus enables a relatively quick assessment of the percentage of cells expressing GFP and is used as a method of assessing efficiency of infection of the Hsp viruses, as the Hsp expressing viruses do not contain a visual marker; the assumption made is cells infected with the same amount of virus and at the same m.o.i, will result in the same transduction efficiency.

Cells were infected at an m.o.i (multiplicity of infection) of ten in figure 5.1a and 5.1b.

(a)

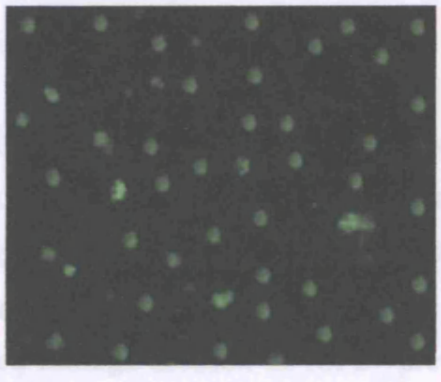


**Figure 5.1a** Representative image of DRGs infected with GFP virus

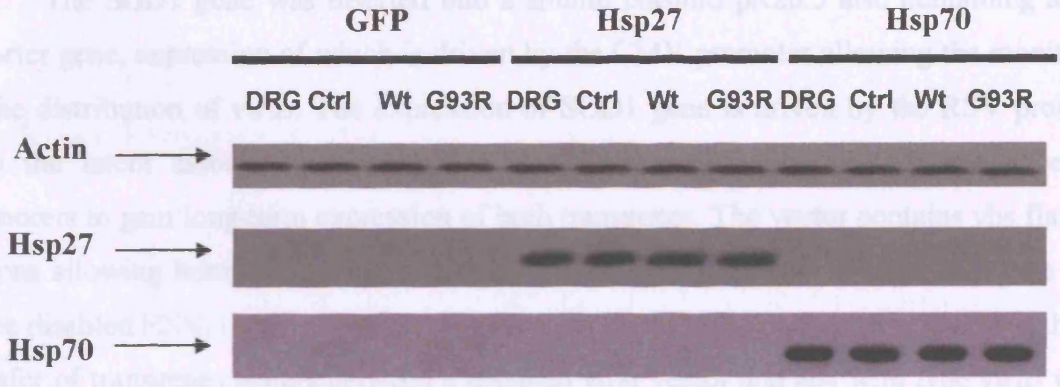



**Figure 5.1b Heat shock protein over expression in rat DRGs expressing wild type or mutant SOD1 at 24h following infection with recombinant HSV-based vectors expressing GFP, Hsp27 or Hsp70.**

Control DRGs, GFP control, wt and mutant G93R-SOD1 expressing cells were infected at an m.o.i of 10 with recombinant viruses over-expressing either GFP, Hsp27 or Hsp70; after which western blotting was carried out with antibodies: anti-β-actin, anti-hsp27 and anti-hsp70 antibodies. There is no significant induction of Hsp expression as a result of infection with the virus itself and this can be seen with the GFP control virus, however, there is high expression of the transgenes, all driven by the CMV-IE promoter.

### 5.3.3 SOD HSV Virus

Having demonstrated using cell lines, that overexpression of wild type SOD1 was able to provide a protective effect against a variety of damaging stimuli, a system was developed for manipulating SOD1 expression efficiently both in cultured cells and ultimately *in vivo*. The human SOD1 gene was inserted into a nonvirulent strain (1764) of HSV. This virus was disabled through deletion of ICP27 preventing lytic replication and making the virus avirulent *in vivo* but requiring complementation for growth in culture. Further disablement was achieved by deleting nonessential HSV-1 genes ICP34.5 and inserting an inactivating insertion in VMW65 thereby abolishing its ability to transactivate immediate early genes but allowing encoding of its essential structural protein.

The SOD1 gene was inserted into a shuttle plasmid pR20.5 also containing a GFP reporter gene, expression of which is driven by the CMV promoter allowing the monitoring of the distribution of virus. The expression of SOD1 gene is driven by the RSV promoter with the latent associated transcript LAT P2 region (from HSV-1) between the two promoters to gain long-term expression of both transgenes. The vector contains vhs flanking regions allowing homologous recombination into the virion host shutoff protein gene (vhs) in the disabled HSV-1 viral construct thereby further disabling the virus and ensuring that no transfer of transgenes occurs between a disabled viral vector and any wild type virus which it may encounter.

Following infection of cultured ND7 cells with the viruses, recombinant SOD1 transgene insertion was confirmed by Southern blot analysis and the expression of protein was confirmed by Western blotting (Fig. 5.2). The protein product corresponding to human SOD1 was seen to be present in the two SOD1 infected cells and the white blood cell sample extract.

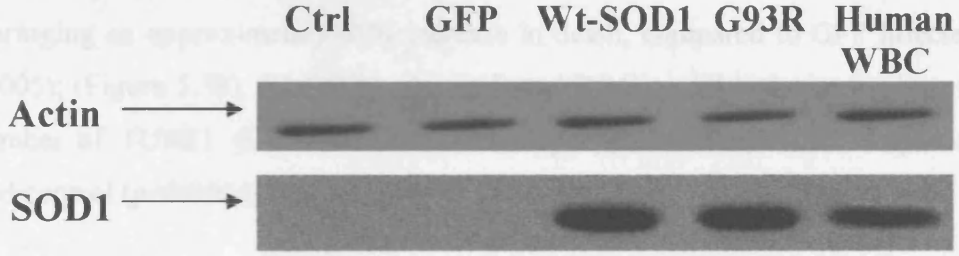



**Figure 5.2** Western blot confirmation of SOD1 protein in ND7 cells. Levels of SOD1 expression in the GFP-control infected ND7 cells (lane 2), wt-SOD1 infected ND7 cells (lane 3), G93R-mutant SOD1 infected ND7 cells (lane 4), and human white blood cells (lane 5) compared to the control ND7 cells (lane 1).

### 5.3.4 Neuroprotective effect of viral delivery of SOD1

Total cell death and number of apoptotic cells after were assessed by trypan blue exclusion and TUNEL assay respectively, at time 24h following exposure to stress. DRG

neuronal cells were either mock infected (no virus) or infected with HSV-1 vector GFP virus (control) or expressing wt-SOD1 or G93R mutant virus.

## 5.4 Responses of SOD Virus infected Rat DRG Cells to Death-inducing Stimuli

### 5.4.1 NGF withdrawal

Infection of rat DRGs with G93R mutant SOD1 virus brought about an approximately 60% increase in death, compared to GFP infected control ($p<0.0005$); (Figure 5.3a). Wt-SOD1 virus infected DRGs exhibited a protective effect with total cell death reduced by approximately 59% compared to GFP infected control ($p<0.0005$).

TUNEL analysis provided similar results with infection of DRGs with G93R mutant SOD1 virus bringing an approximately 65% increase in death, compared to GFP infected control ($p<0.0005$); (Figure 5.3b). Wt-SOD1 virus infected DRGs exhibited a protective effect with the number of TUNEL positive cells reduced by approximately 62% compared to GFP infected control ($p<0.0005$).

Figure 5.3a

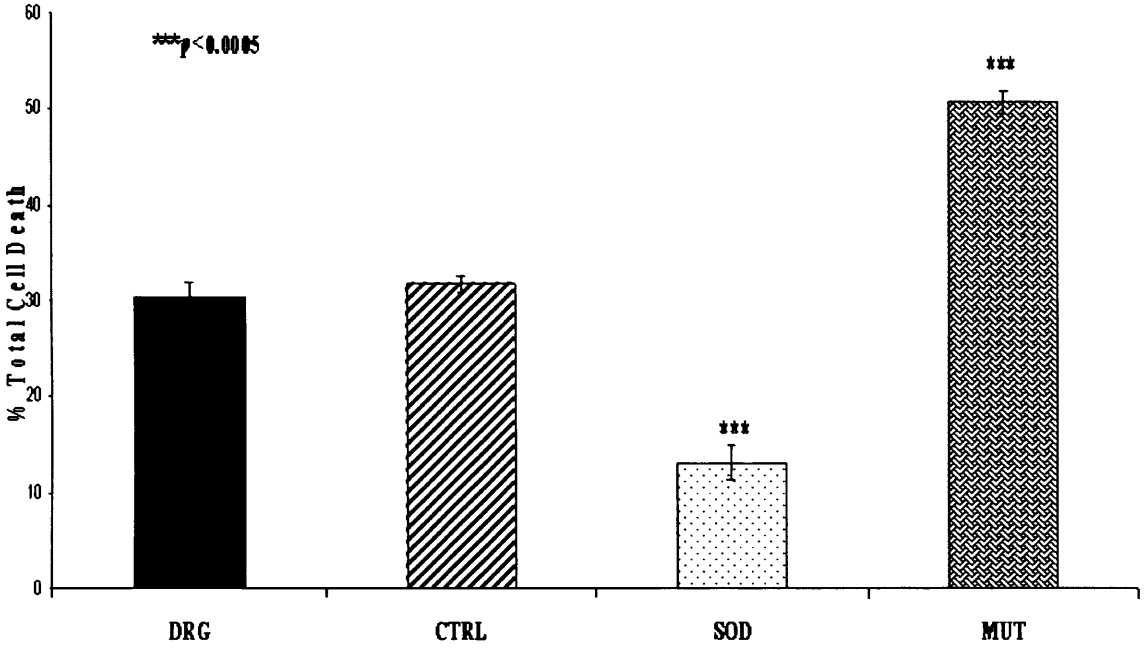



**Figure 5.3 Effect of NGF withdrawal on cell death in control, wild type or mutant SOD1 virus infected rat DRGs**

**a.** Rat DRG cell death following 24h of NGF withdrawal after infection with HSV vectors expressing wt-SOD1 or G93R mutant. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of treatment of NGF withdrawal of rat DRG cells infected with wt or G93R-mutant SOD1 or GFP control. Cells were infected with SOD viruses 16h prior to stress. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=4. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p<0.0005$). *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

Figure 5.3b

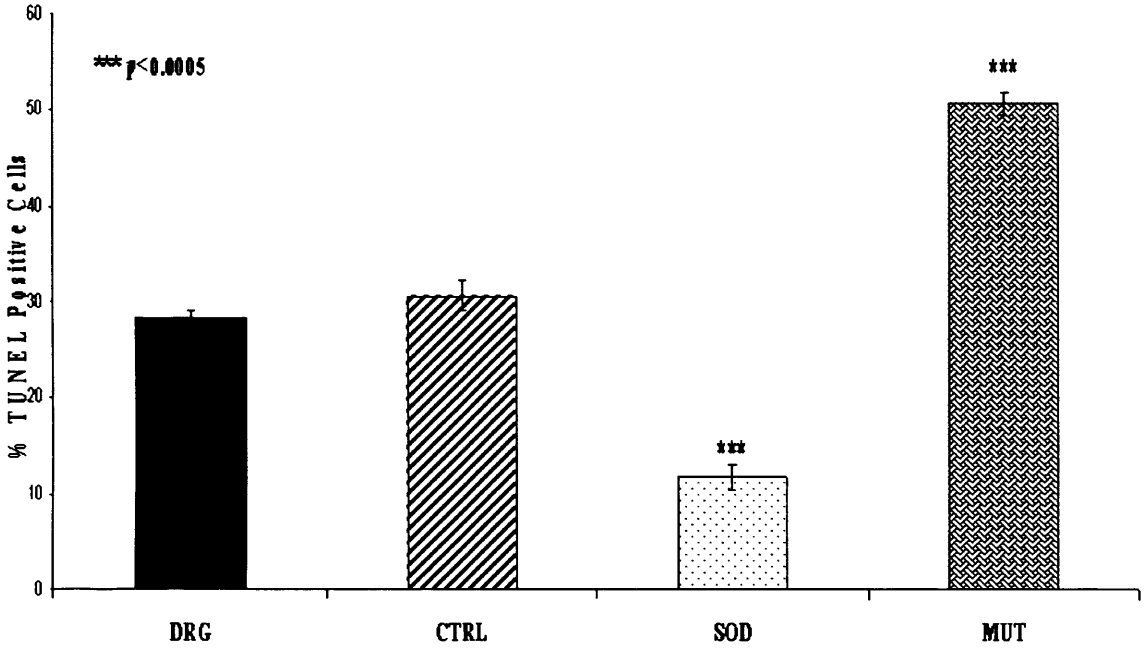



**Figure 5.3 Effect of NGF withdrawal on cell death in control, wild type or mutant SOD1 virus infected rat DRGs**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were infected with viruses to over-express wt-SOD1, G93R-mutant or GFP 16h prior to treatment of NGF withdrawal for 24h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=4) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

### 5.4.2 IFN-γ administration

G93R mutant infected DRGs exhibited an approximately 63% increase in death compared to GFP infected control ($p<0.0005$); (Figure 5.4a). Wt-SOD1 infected DRGs again exhibited a protective effect with a reduction in death by approximately 73% compared to GFP infected control ($p<0.0005$).

TUNEL analysis revealed the G93R mutant infected DRGs exhibited an approximately 63% increase in the number of TUNEL positive cells compared to GFP infected control ($p<0.0005$); (Figure 5.4b). Wt-SOD1 infected DRGs again exhibited a protective effect with a reduction in the number of TUNEL positive cells by approximately 75% compared to GFP infected control ($p<0.0005$).

Figure 5.4a

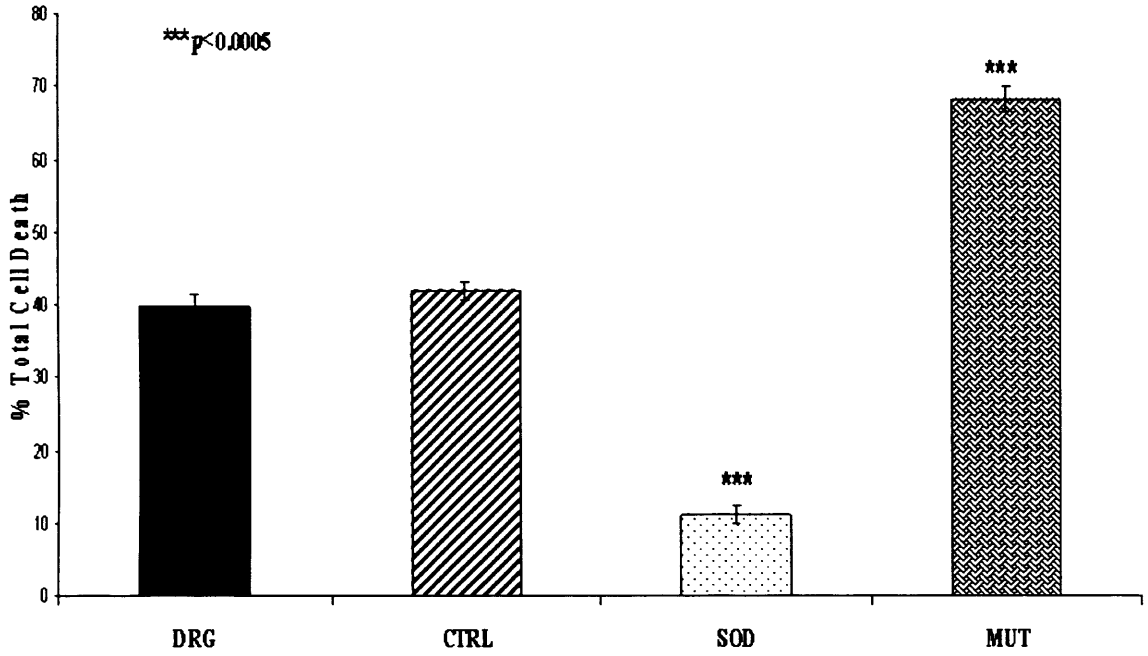



**Figure 5.4 Effect of IFN-γ on cell death in control, wild type or mutant SOD1 virus infected rat DRGs**

**a.** Rat DRG cell death following 24h of treatment with 50ng/ml IFN-γ after infection with HSV vectors expressing wt-SOD1 or G93R mutant. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of treatment of 50ng/ml IFN-γ of rat DRG cells infected with wt or G93R-mutant SOD1 or GFP control. Cells were infected with SOD viruses 16h prior to stress. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=4. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p<0.0005$). *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

Figure 5.4b

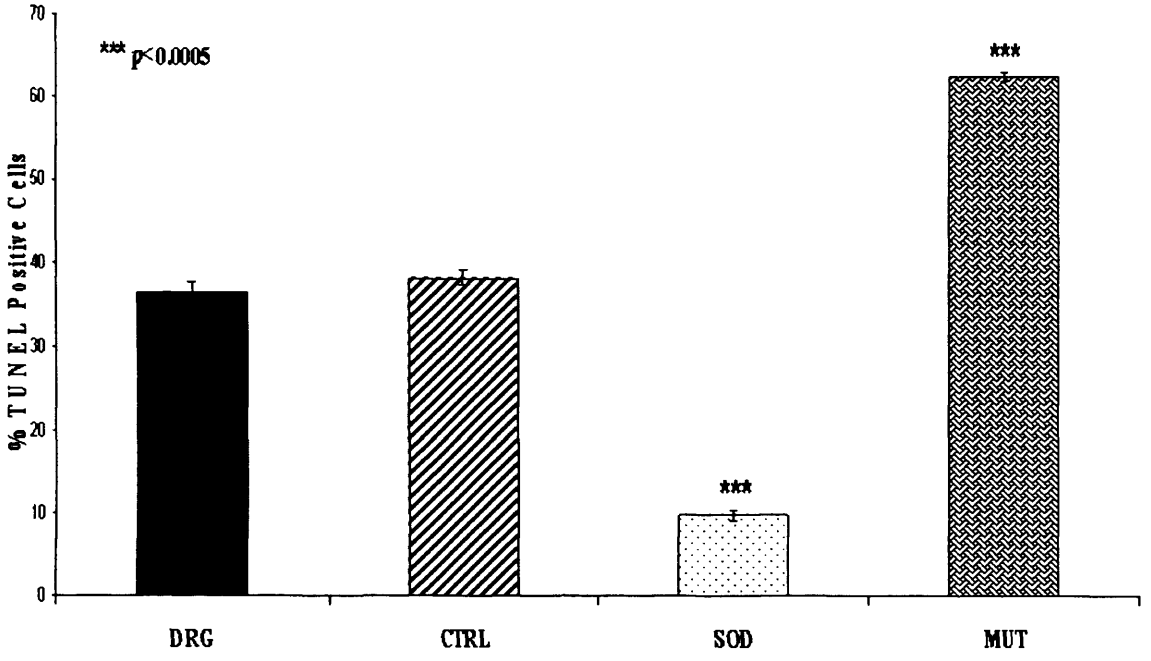



**Figure 5.4 Effect of IFN-γ on cell death in control, wild type or mutant SOD1 virus infected rat DRGs**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were infected with viruses to over-express wt-SOD1, G93R-mutant or GFP 16h prior to treatment of 50ng/ml IFN-γ for 24h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=4) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

### 5.4.3 Staurosporine treatment

G93R mutant infected DRGs exposed to 1μM staurosporine exhibited an approximately 60% increase in total cell death compared to GFP infected control($p<0.0005$); (Figure 5.5a). Wt-SOD1 infected DRGs provided a protective effect against the stress with a reduction on death of approximately 58% compared to GFP infected control ($p<0.0005$).

TUNEL analysis provided similar results with G93R mutant infected DRGs exposed to 1μM staurosporine exhibiting an approximately 63% increase in the number of TUNEL positive cells compared to GFP infected control($p<0.0005$); (Figure 5.5b). Wt-SOD1 infected DRGs provided a protective effect against the stress with a reduction in the number of TUNEL positive cells by approximately 60% compared to GFP infected control ($p<0.0005$).

Figure 5.5a

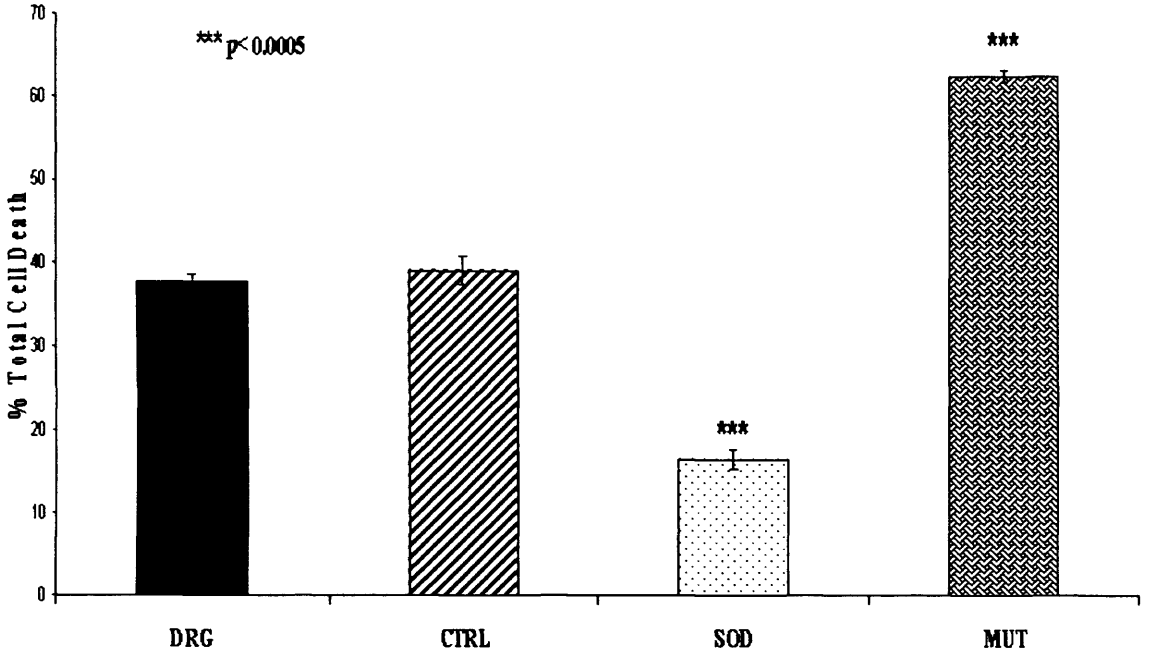



**Figure 5.5 Effect of Staurosporine on cell death in control, wild type or mutant SOD1 virus infected rat DRGs**

**a.** Rat DRG cell death following 24h of 1μM staurosporine administration after infection with HSV vectors expressing wt-SOD1 or G93R mutant. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of treatment of 1μM staurosporine of rat DRG cells infected with wt or G93R-mutant SOD1 or GFP control. Cells were infected with SOD viruses 16h prior to stress. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=4. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p<0.0005$). *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

Figure 5.5b

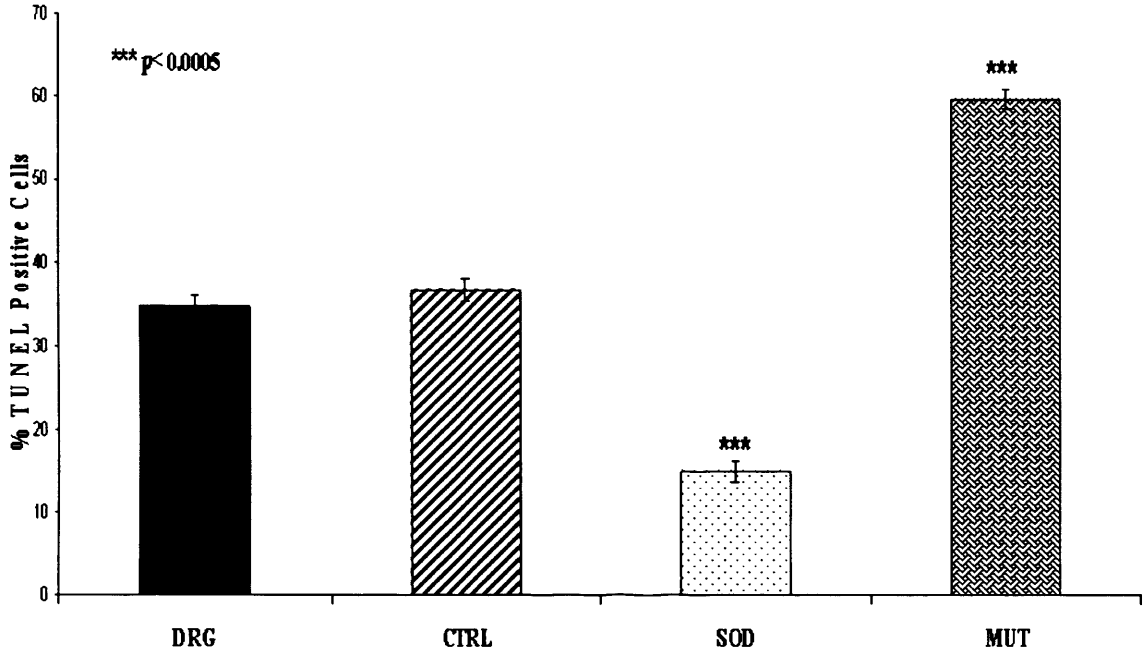



**Figure 5.5 Effect of Staurosporine on cell death in control, wild type or mutant SOD1 virus infected rat DRGs**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were infected with viruses to over-express wt-SOD1, G93R-mutant or GFP 16h prior to treatment of 1μM staurosporine for 24h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=4) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

## 5.5 Responses of SOD Virus infected wt-mice DRG Cells to Death-inducing Stimuli

### 5.5.1 NGF withdrawal

Wt-mice DRGs infected with G93R mutant SOD virus exhibited enhanced cell death as was observed in previous experiments. G93R mutant cells exhibited a 63% increase in cell death compared to control GFP ($p<0.0005$); (Figure 5.6a). The wt-SOD1 infected DRGs when exposed to stress of NGF withdrawal were protected. Wt-SOD1 infected DRGs showed a 56% decrease in cell death compared to GFP control ($p<0.0005$).

TUNEL analysis revealed wt-mice DRGs infected with G93R mutant SOD virus exhibited enhanced cell death similar to that observed with trypan blue analysis. G93R mutant cells exhibited a 68% increase in the number of TUNEL positive cells compared to control GFP ($p<0.0005$); (Figure 5.6b). The wt-SOD1 infected DRGs when exposed to stress of NGF withdrawal were protected. Wt-SOD1 infected DRGs showed a 61% decrease in the number of TUNEL positive cells compared to GFP control ($p<0.0005$).

Figure 5.6a

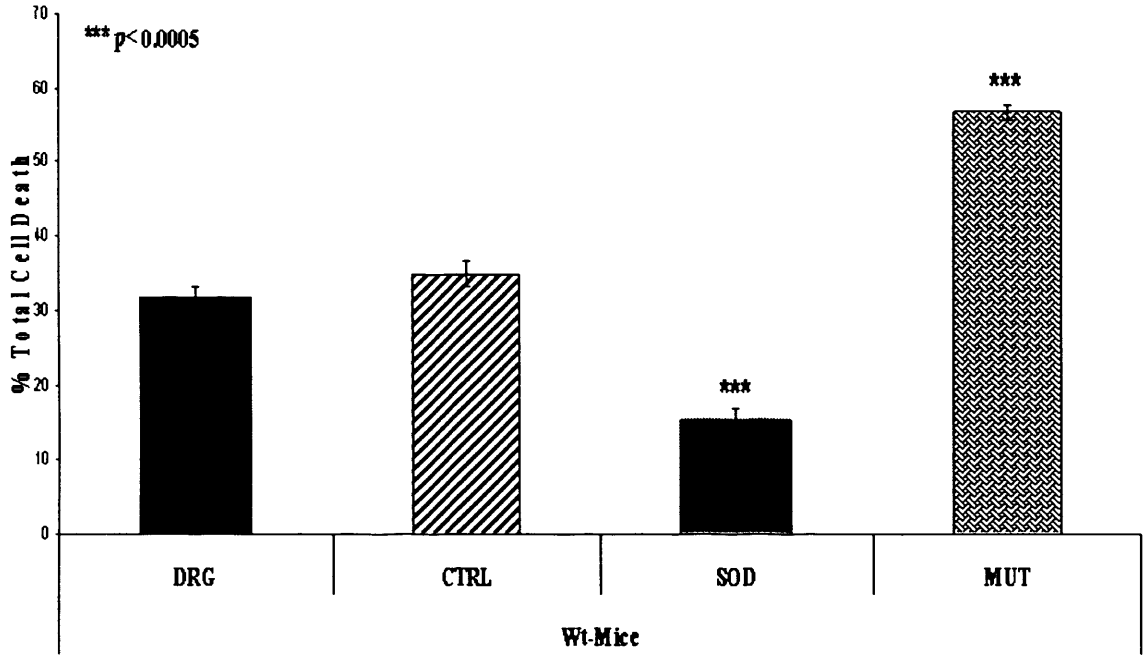



**Figure 5.6 Effect of NGF withdrawal on cell death in control, wild type or mutant SOD1 virus infected wt-mice DRGs**

**a.** Wt-mice DRG cell death following 24h of NGF withdrawal after infection with HSV vectors expressing wt-SOD1 or G93R mutant. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of treatment of NGF withdrawal of wt-mice DRG cells infected with wt or G93R-mutant SOD1 or GFP control. Cells were infected with SOD viruses 16h prior to stress. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=4. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p<0.0005$). *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

Figure 5.6b

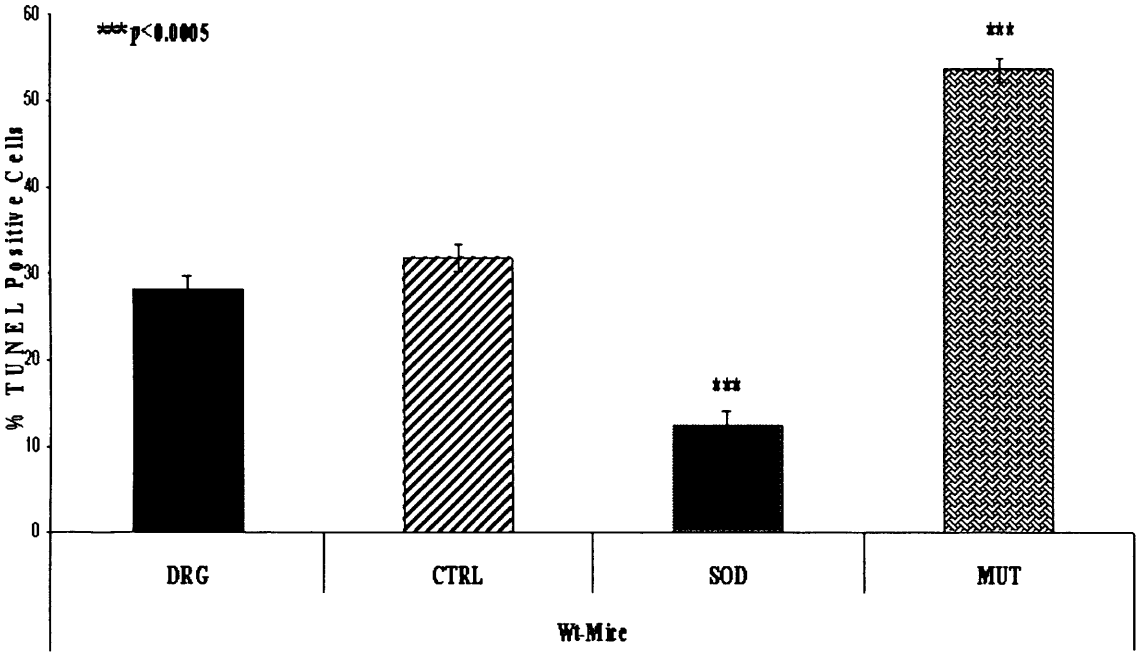



**Figure 5.6 Effect of NGF withdrawal on cell death in control, wild type or mutant SOD1 virus infected wt-mice DRGs**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were infected with viruses to over-express wt-SOD1, G93R-mutant or GFP 16h prior to treatment of NGF withdrawal for 24h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=4) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

### 5.5.2 IFN-γ administration

Wt-mice DRGs infected with mutant G93R virus and then exposed to stress of IFN-γ exhibited increased cell death with death rising by approximately 57% compared to GFP infected control ($p<0.0005$). Wt-SOD1 infected DRGs were protected from the stress with cells exhibiting a 63% reduction in cell death compared to GFP infected control ($p<0.0005$); (Figure 5.7a).

TUNEL analysis revealed wt-mice DRGs infected with mutant G93R virus and then exposed to stress of IFN-γ exhibited an increase in the number of TUNEL positive cells with the number rising by approximately 62% compared to GFP infected control ($p<0.0005$). Wt-SOD1 infected DRGs were protected from the stress with cells exhibiting a 68% reduction in the number of TUNEL positive cells compared to GFP infected control ($p<0.0005$); (Figure 5.7b).

Figure 5.7a

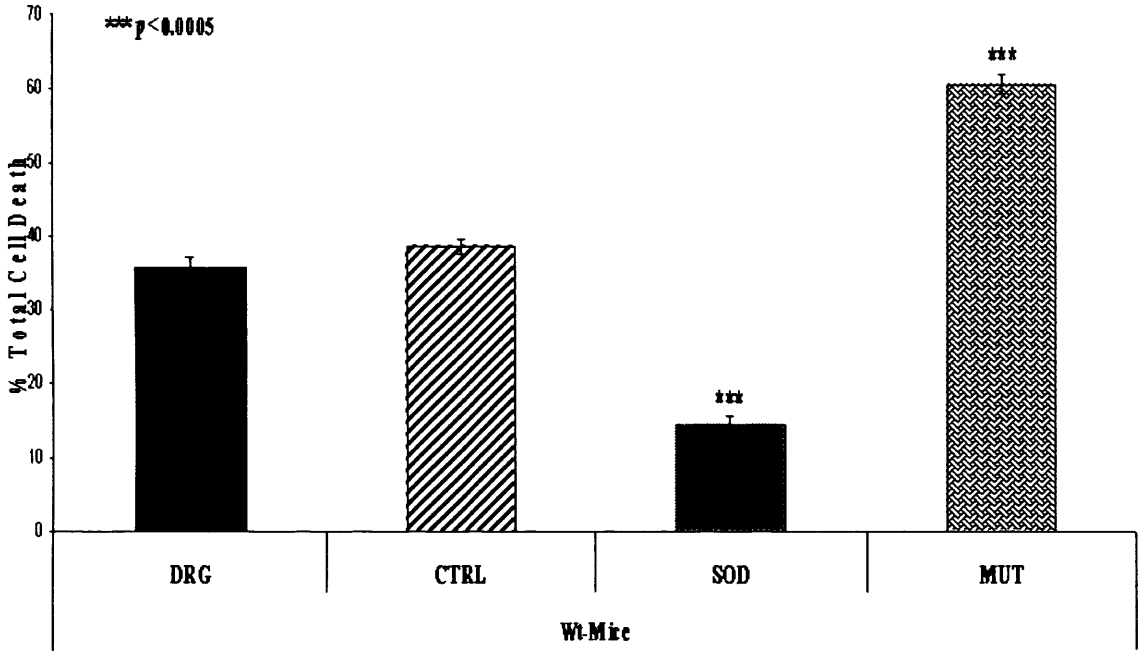



**Figure 5.7 Effect of IFN-γ on cell death in control, wild type or mutant SOD1 virus infected wt-mice DRGs**

**a.** Wt-mice DRG cell death following 24h of treatment with 50ng/ml IFN-γ after infection with HSV vectors expressing wt-SOD1 or G93R mutant. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of treatment of 50ng/ml IFN-γ of wt-mice DRG cells infected with wt or G93R-mutant SOD1 or GFP control. Cells were infected with SOD viruses 16h prior to stress. Bars represent mean $\pm$ S.D calculated for triplicate counts per sample (n), n=4. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p<0.0005$). *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

Figure 5.7b

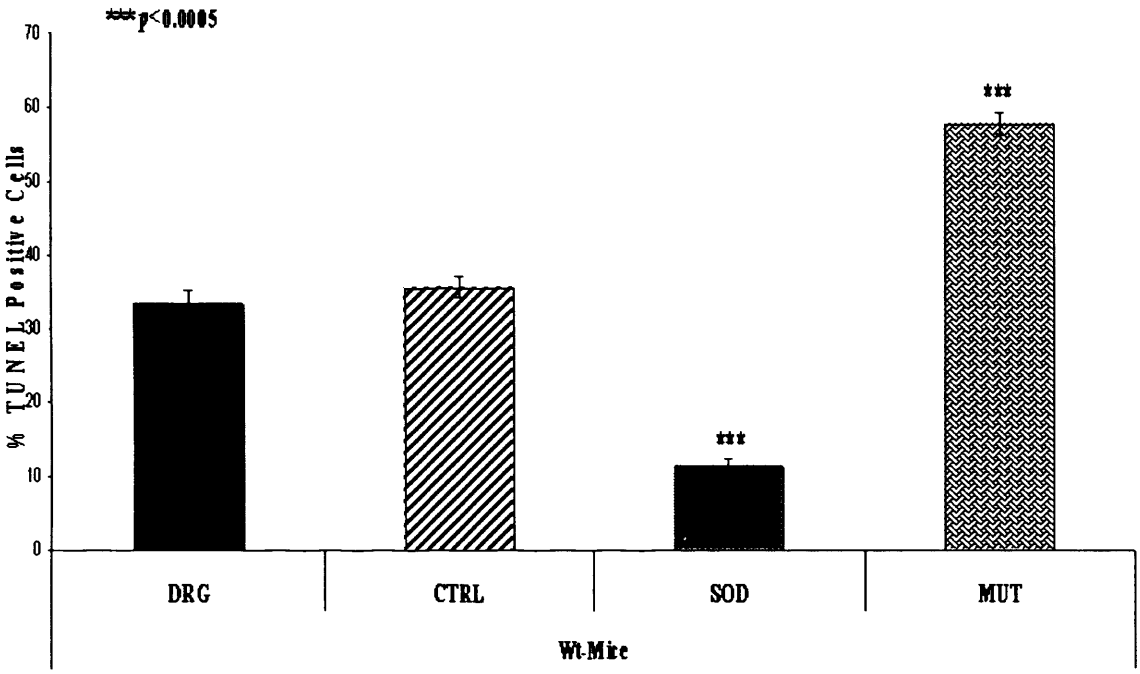



**Figure 5.7 Effect of IFN-γ on cell death in control, wild type or mutant SOD1 virus infected wt-mice DRGs**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were infected with viruses to over-express wt-SOD1, G93R-mutant or GFP 16h prior to treatment of 50ng/ml IFN-γ for 24h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=4) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

### 5.5.3 Staurosporine treatment

G93R mutant virus infected mice DRGs exposed to staurosporine showed an 81% increase in total cell death compared to GFP infected control ($p<0.0005$). Once again mice DRGs infected with wt-SOD1 virus were protected with death being reduced by approximately 56% compared to GFP control ($p<0.0005$); (Figure 5.8a).

These results were confirmed by TUNEL analysis with the G93R mutant virus infected wt-mice DRGs exposed to staurosporine exhibiting a 88% increase in the number of TUNEL positive cells compared to GFP infected control ($p<0.0005$). Once again wt-mice DRGs infected with wt-SOD1 virus were protected with the number of TUNEL positive cells being reduced by approximately 61% compared to GFP control ($p<0.0005$); (Figure 5.8b).

Figure 5.8a

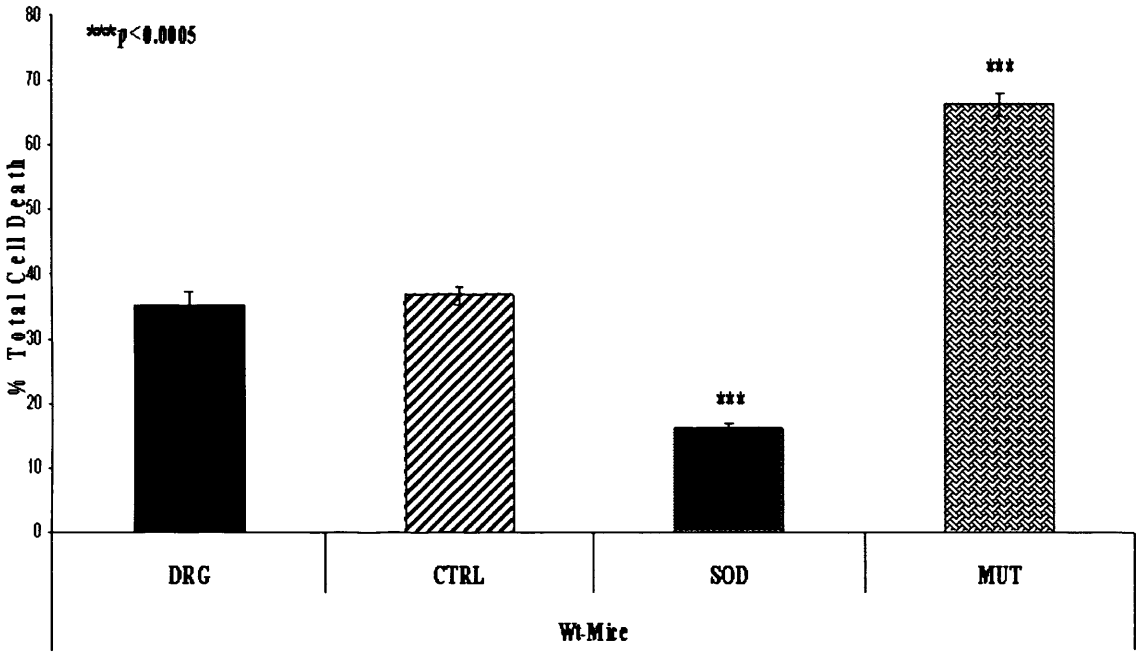



**Figure 5.8 Effect of Staurosporine on cell death in control, wild type or mutant SOD1 virus infected wt-mice DRGs**

**a.** Wt-mice DRG cell death following 24h of 1μM staurosporine administration after infection with HSV vectors expressing wt-SOD1 or G93R mutant. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of treatment of 1μM staurosporine of wt-mice DRG cells infected with wt or G93R-mutant SOD1 or GFP control. Cells were infected with SOD viruses 16h prior to stress. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=4. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p<0.0005$). *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

Figure 5.8b

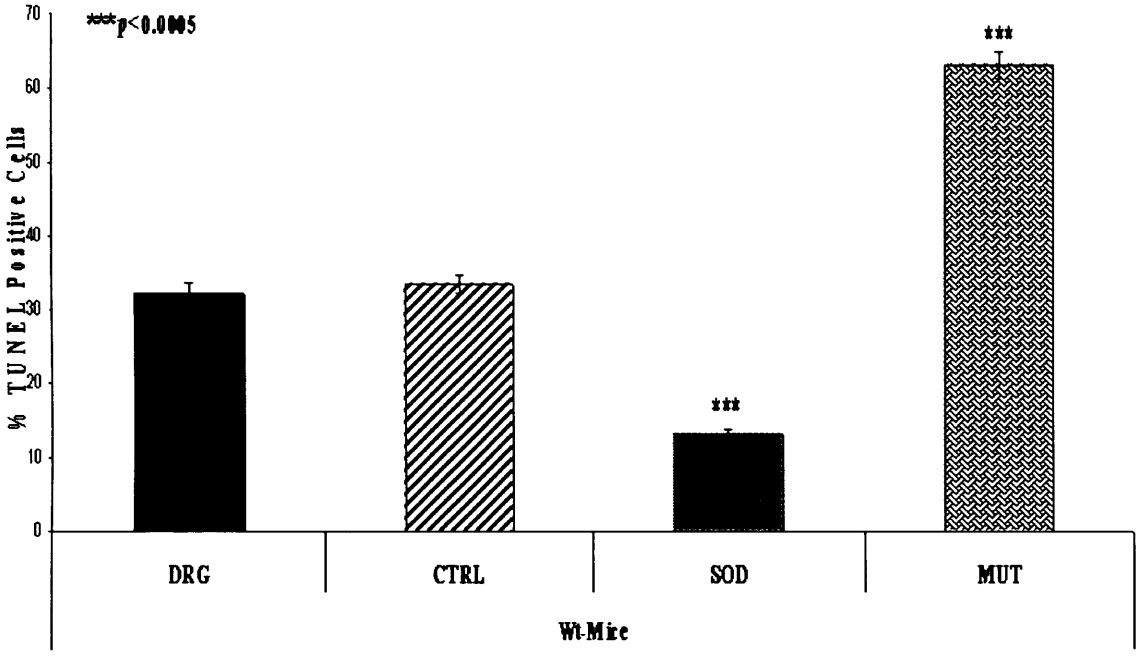



**Figure 5.8 Effect of Staurosporine on cell death in control, wild type or mutant SOD1 virus infected wt-mice DRGs**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were infected with viruses to over-express wt-SOD1, G93R-mutant or GFP 16h prior to treatment of 1μM staurosporine for 24h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=4) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

## 5.6 Responses of the SOD virus infected DRG Neuronal Cells to Stress following Exogenous Heat Shock Protein Over-expression

Having characterised the effect of wt-SOD1, G93R-SOD1 mutant and its GFP control in the rat and mouse DRG neuronal cells, the next step was to demonstrate that Hsps can be over expressed in these cells through use of viral vectors and then to confirm the potential relevance of the results obtained in chapter 4 to the *in vivo* situation and utilise this system to test whether Hsps can confer neuroprotection against the toxic effects of the G93R-SOD1 mutant. This section therefore describes the results obtained following experimentation into the protective effect of individual Hsps as well as Hsps in combination, in this model system. As with chapters 3 and 4, the findings for the various stresses are presented below the corresponding stress and cell death was assessed by trypan blue exclusion assay and TUNEL analysis.

### 5.6.1 Infection of SOD virus infected DRGs with Hsp Virus

In order to investigate the protective effects of Hsps further, we infected primary cultures of DRGs with wt-SOD1, G93R-SOD1 mutant or control GFP virus along with either Hsp27, Hsp70 or the combination of Hsp27 and Hsp70. The primary cultures were then subjected 16h post infection to NGF withdrawal, IFN-γ or staurosporine administration. The results paralleled the results of the neuronal cell line experiments with wt-SOD1 protecting the DRGs and reducing death upon exposure to stress and G93R mutant infected DRGs exhibiting increased death. On infection of G93R mutant infected DRGs with Hsp27 or Hsp70 death was reduced with the combination of Hsp27 and Hsp70 being the most effective in reducing mutant death. Wt-SOD1 infected DRGs exhibited increased death on infection with Hsp27 or Hsp70 as observed in the neuronal cell lines with the dual infection of Hsp27 and Hsp70 reducing death back down toward control levels.

## 5.7 Responses of SOD Virus infected Rat DRG Cells to Stress following Exogenous Heat Shock Protein Over-expression

### 5.7.1 NGF withdrawal

Rat DRGs infected with the G93R mutant virus showed a reduction in death on infection with Hsp virus similar to that observed in the experiments carried out in the neuronal stable cell lines. On infection of G93R mutant DRGs with Hsp27 virus a reduction in death of approximately 52% was observed, compared to GFP infected control ($p<0.0005$); (Figure 5.9a). Hsp70 virus was slightly more effective in reducing the death associated with the G93R mutant, paralleling the findings of the stable cell line experiments, with death being reduced by approximately 63% compared to GFP control ($p<0.0005$). The combination of Hsp27 and Hsp70 was the most effective in reducing the mutant death with death being reduced by approximately 71% compared to GFP control ($p<0.0005$). The wt-SOD1 infected cells exhibited a dramatic increase in cell death on infection with Hsp27 or Hsp70 with death being increased by approximately 102% and 234% respectively compared to GFP infected control ($p<0.0005$). The dual infection of Hsp27 and Hsp70 brought about a reduction in death toward control levels, however death was still up by approximately 54% compared to control GFP ($p<0.0005$). Addition of zVAD reduced total cell death across all cells with the exception of wt-SOD1 infected cells, which exhibited an increase in cell death of approximately 7% compared to control. G93R mutant death was reduced by approximately 70% compared to control ($p<0.0005$) on addition of zVAD and was almost as effective as the dual infection of Hsp27 and Hsp70.

TUNEL analysis confirmed and supported the results obtained by trypan blue analysis. On infection of G93R mutant DRGs with Hsp27 virus a reduction of approximately 54% in the number of TUNEL positive cells was observed, compared to GFP infected control ($p<0.0005$); (Figure 5.9b). Hsp70 virus was slightly more effective on reducing the number of TUNEL positive cells of the G93R mutant with number being reduced by approximately 66% compared to GFP control ($p<0.0005$). The combination of Hsp27 and Hsp70 was the most effective on the G93R mutant, with the number of TUNEL positive cells being reduced by approximately 76% compared to GFP control ($p<0.0005$). The wt-SOD1 infected cells

exhibited an increase in the number of TUNEL positive cells, on infection with Hsp27 or Hsp70, with the number of TUNEL positive cells increasing by approximately 141% and 298% respectively compared to GFP infected control ($p<0.0005$). The dual infection of Hsp27 and Hsp70 on the wt-SOD1 infected DRGs brought about a reduction in the number of TUNEL positive cells toward control levels, however the increase in death was still approximately 80% greater than control GFP ($p<0.0005$). Addition of zVAD reduced the number of TUNEL positive cells across all cells except those infected with wt-SOD1, which exhibited an increase in the number of TUNEL positive cells by approximately 22% compared to control. G93R mutant cells exhibited an approximately 71% ($p<0.0005$) reduction in the number of TUNEL positive cells compared to control on addition of zVAD; this reduction was almost as effective as that observed with the dual Hsp27 and Hsp70 infection.

Figure 5.9a

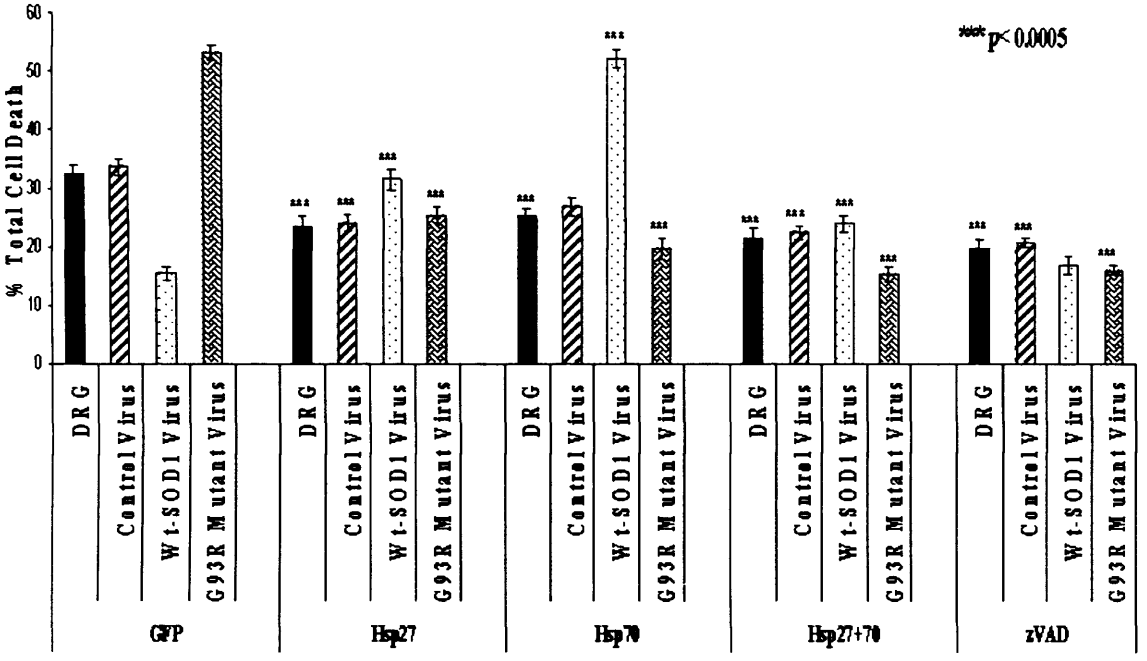



**Figure 5.9 Effect of NGF withdrawal on cell death in control, wild type or mutant SOD1 virus infected rat DRGs**

**a.** Rat DRG cell death following 24h of NGF withdrawal after co-infection with HSV vectors expressing wt-SOD1 or G93R mutant and Hsp27, Hsp70 or Hsp27 and Hsp70. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of treatment of NGF withdrawal of rat DRG cells infected with wt or G93R-mutant SOD1 or GFP control and Hsp27, Hsp70 or Hsp27 and Hsp70. Cells were co-infected with SOD and Hsp viruses 16h prior to stress. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=4. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p$<0.0005). *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

Figure 5.9b

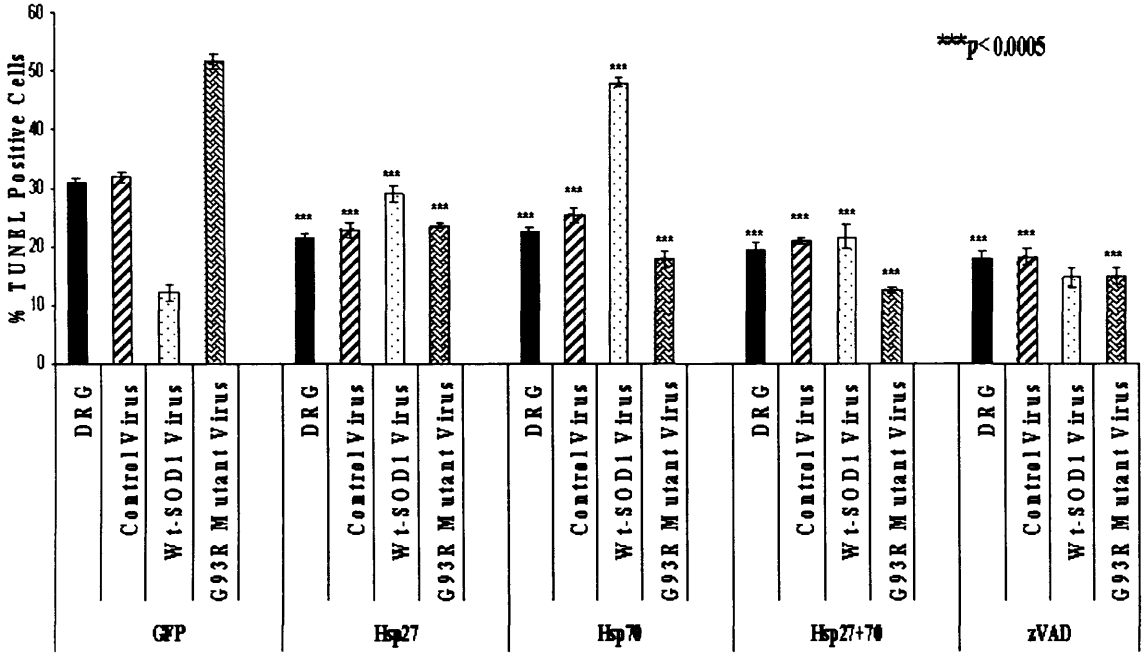



**Figure 5.9 Effect of NGF withdrawal on cell death in control, wild type or mutant SOD1 virus infected rat DRGs**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were co-infected with viruses to over-express wt-SOD1, G93R-mutant or GFP and Hsp27, Hsp70 or Hsp27 and Hsp70 16h prior to treatment of NGF withdrawal for 24h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=4) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

### 5.7.2 IFN-γ administration

G93R mutant infected DRGs exhibited an approximately 59% reduction in death on infection with Hsp27 compared to GFP infected control ($p<0.0005$). On infection with Hsp70 the G93R mutant infected DRGs exhibited an approximately 71% reduction in death compared to GFP infected control ($p<0.0005$); (Figure 5.10a). The dual infection of Hsp27 and Hsp70 brought about the greatest protection by reducing the death of the G93R mutant infected DRGs by approximately 79% compared to GFP infected control ($p<0.0005$). Infection of the wt-SOD1 infected cells with Hsp27 or Hsp70 led to an increase in death with Hsp70 infection bringing about a larger increase in death than Hsp27. On infection of wt-SOD1 infected DRGs with Hsp27 an increase in cell death of approximately 282% was observed and an approximately 312% increase observed on infection with Hsp70, compared to GFP control ($p<0.0005$). The dual infection of wt-SOD1 infected DRGs with Hsp27 and Hsp70 brought about a reduction in death towards control levels but still exhibited an approximately 79% increase over GFP infected control ($p<0.0005$). Total cell death was reduced across all cells except those infected with wt-SOD1, which exhibited an approximately 19% increase in death compared to control. Addition of zVAD reduced the G93R mutant death by approximately 78% compared to control ($p<0.0005$) and was almost as effective at reducing the mutant death as the Hsp27 and Hsp70 dual infection.

TUNEL analysis confirmed the trypan blue assay results with G93R mutant infected DRGs exhibiting an approximately 61% reduction in the number of TUNEL positive cells on infection with Hsp27 compared to GFP infected control ($p<0.0005$). On infection with Hsp70 the G93R mutant infected DRGs exhibited an approximately 71% reduction in the number of TUNEL positive cells compared to GFP infected control ($p<0.0005$); (Figure 5.10b). The dual infection of Hsp27 and Hsp70 brought about the greatest protection by reducing the number of TUNEL positive cells, of the G93R mutant, by approximately 81% compared to GFP infected control ($p<0.0005$). Infection of the wt-SOD1 infected cells with Hsp27 or Hsp70, led to an increase in the number of TUNEL positive cells, with Hsp70 infection bringing about a larger increase in the number of TUNEL positive cells than Hsp27. On infection of wt-SOD1 infected DRGs with Hsp27 or Hsp70 an increase in the number of TUNEL positive cells by approximately 293% and 331% was observed

respectively compared to GFP control($p<0.0005$). The dual infection of wt-SOD1 infected DRGs with Hsp27 and Hsp70 brought about a reduction in the number of TUNEL positive cells down towards control levels but death was still approximately 88% higher than GFP infected control ($p<0.0005$). Addition of zVAD to the G93R-mutant infected cells reduced the number of TUNEL positive cells by approximately 80% compared to control ($p<0.0005$) with wt-SOD1 cells exhibiting an approximately 12% increase in the number of TUNEL positive cells compared to control.

Figure 5.10a

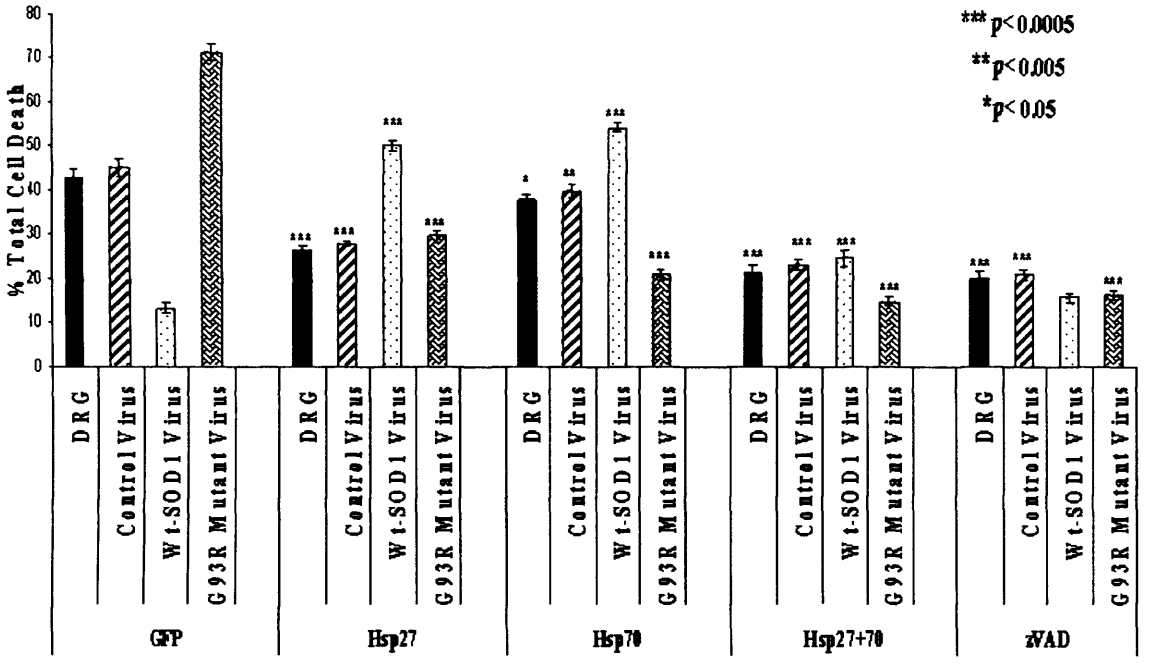



**Figure 5.10 Effect of IFN-γ on cell death in control, wild type or mutant SOD1 virus infected rat DRGs**

**a.** Rat DRG cell death following 24h of treatment with 50ng/ml IFN-γ after co-infection with HSV vectors expressing wt-SOD1 or G93R mutant and Hsp27, Hsp70 or Hsp27 and Hsp70. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of treatment of 50ng/ml IFN-γ of rat DRG cells co-infected with wt or G93R-mutant SOD1 or GFP control and Hsp27, Hsp70 or Hsp27 and Hsp70. Cells were co-infected with SOD and Hsp viruses 16h prior to stress. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=4. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p$<0.0005). *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

Figure 5.10b

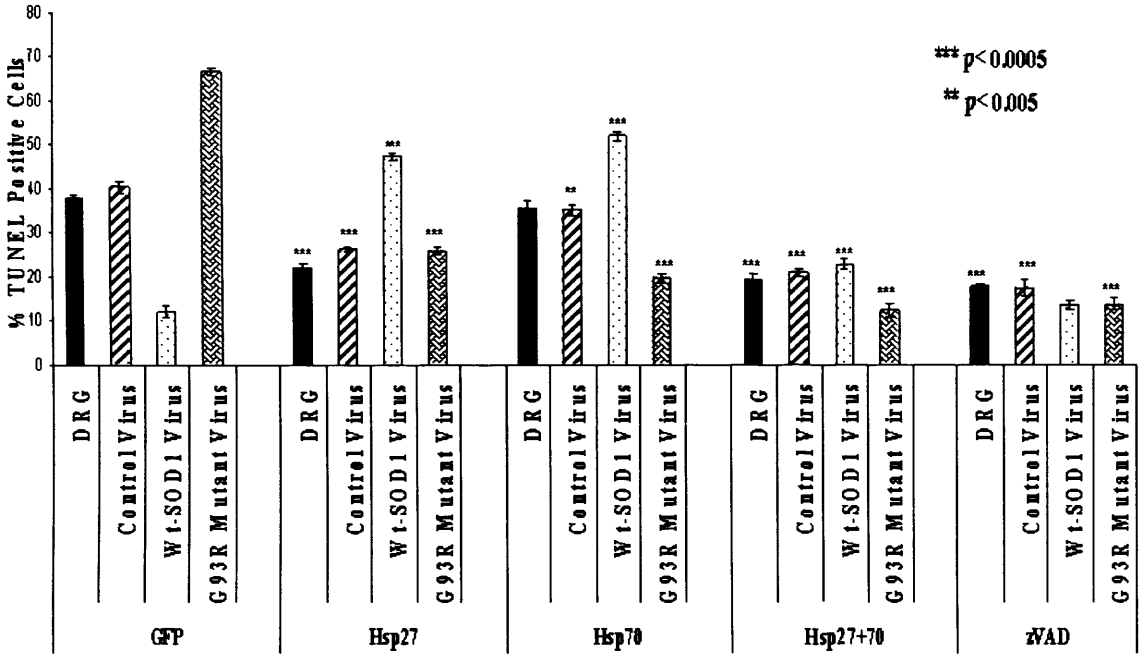



**Figure 5.10 Effect of IFN-γ on cell death in control, wild type or mutant SOD1 virus infected rat DRGs**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were co-infected with viruses to over-express wt-SOD1, G93R-mutant or GFP and Hsp27, Hsp70 or Hsp27 and Hsp70 16h prior to treatment of 50ng/ml IFN-γ for 24h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=4) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

### 5.7.3 Staurosporine treatment

DRGs infected with G93R mutant virus exhibited a reduction of approximately 58% on infection with Hsp27 compared to GFP infected control ($p<0.0005$); (Figure 5.11a). Hsp70 infection of the G93R mutant cells brought about a slightly greater protection with death being reduced by approximately 71% compared to GFP control ($p<0.0005$). The dual infection of Hsp27 and Hsp70 was the most effective in reducing the G93R-mutant death with death being reduced by approximately 77% compared to GFP infected control ($p<0.0005$). The wt-SOD1 infected cells once infected with Hsp27 or Hsp70 exhibited a dramatic increase in cell death with death increasing by approximately 75% and 180% respectively compared to GFP control ($p<0.0005$). On dual infection with Hsp27 and Hsp70 death in the wt-SOD1 infected DRGs was brought back down to control GFP levels. However it was still approximately 31% higher than control GFP cells ($p<0.0005$). Once again addition of zVAD reduced G93R-mutant death almost as efficiently as the dual infection with Hsp27 and Hsp70 with death being reduced by approximately 70% compared to control ($p<0.0005$). Wt-SOD1 infected cells on the other hand exhibited a 10% increase in cell death compared to control.

TUNEL analysis confirmed the trypan findings with DRGs infected with G93R mutant virus exhibiting a reduction of approximately 61% in the number of TUNEL positive cells on infection with Hsp27 compared to GFP infected control ($p<0.0005$); (Figure 5.11b). On infection of the G93R mutant cells with Hsp70 the number of TUNEL positive cells were reduced by approximately 73% compared to GFP control ($p<0.0005$). The dual infection of Hsp27 and Hsp70 reduced the number of TUNEL positive cells of the G93R mutant by approximately 80% compared to GFP infected control ($p<0.0005$). Wt-SOD1 infected cells once infected with Hsp27 or Hsp70 exhibited an increase in the number of TUNEL positive cells with the number increasing by approximately 77% and 192% respectively compared to GFP control ($p<0.0005$). Dual infection with Hsp27 and Hsp70 of the wt-SOD1 DRGs reduced the number of TUNEL positive cells back down to control GFP levels, however the number of TUNEL positive cells was still approximately 26% higher than control GFP ($p<0.05$). Addition of zVAD to G93R mutant infected cells reduced the number of TUNEL positive cells by approximately 71% ($p<0.0005$) compared to control, with wt-SOD1

infected cells exhibiting a slight increase in the number of TUNEL positive cells by 6% compared to control.

Figure 5.11a

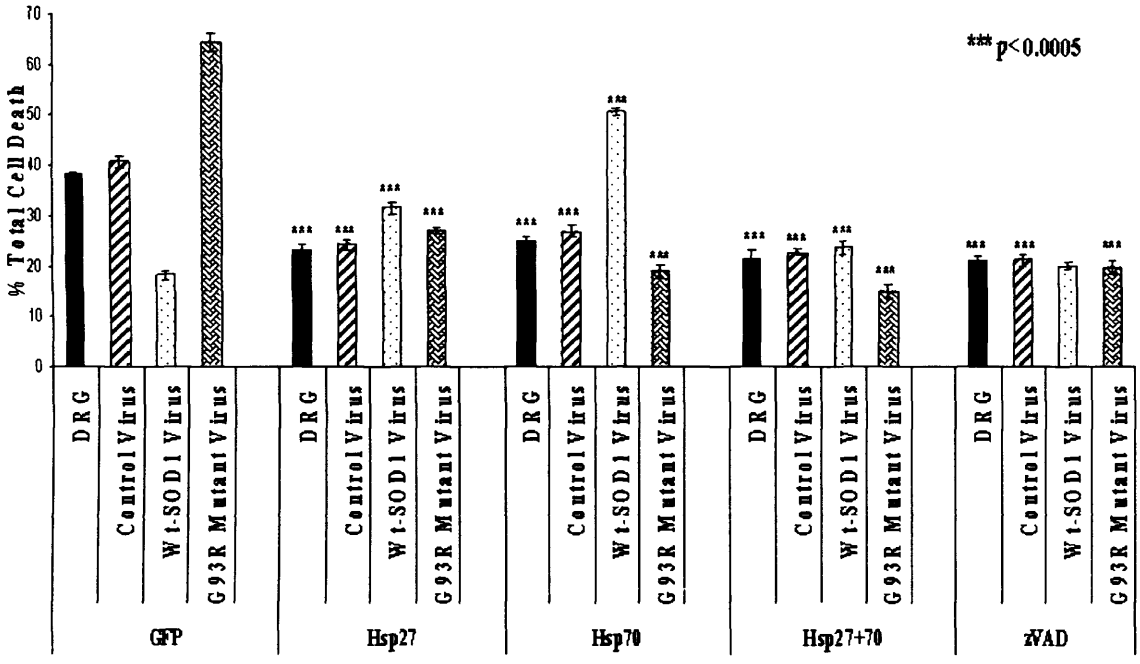



**Figure 5.11 Effect of Staurosporine on cell death in control, wild type or mutant SOD1 virus infected rat DRGs**

**a.** Rat DRG cell death following 24h of 1μM staurosporine administration after co-infection with HSV vectors expressing wt-SOD1 or G93R mutant and Hsp27, Hsp70 or Hsp27 and Hsp70. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of treatment of 1μM staurosporine of rat DRG cells co-infected with wt or G93R-mutant SOD1 or GFP control and Hsp27, Hsp70 or Hsp27 and Hsp70. Cells were co-infected with SOD and Hsp viruses 16h prior to stress. Bars represent mean $^{+}$S.D. calculated for triplicate counts per sample (n), n=4. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p$<0.0005). *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

Figure 5.11b

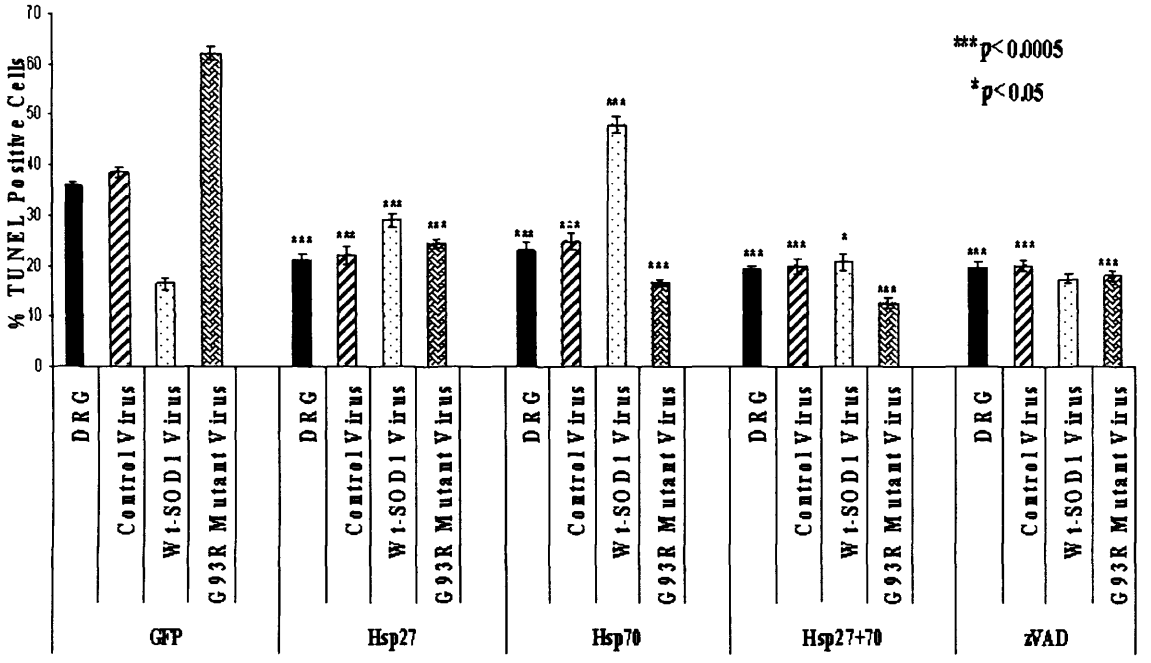



**Figure 5.11 Effect of Staurosporine on cell death in control, wild type or mutant SOD1 virus infected rat DRGs**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were co-infected with viruses to over-express wt-SOD1, G93R-mutant or GFP and Hsp27, Hsp70 or Hsp27 and Hsp70 16h prior to treatment of 1μM staurosporine for 24h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=4) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

## 5.8 Responses of SOD Virus infected wt-mice DRG Cells to Stress following Exogenous Heat Shock Protein Over-expression

### 5.8.1 NGF withdrawal

G93R mutant SOD infected mouse DRG exhibited a reduction in death by approximately 43% and 61% on infection with Hsp27 and Hsp70 respectively compared to GFP infected control ($p<0.0005$); (Figure 5.12a). On dual infection of Hsp27 and Hsp70 the G93R infected cells exhibited the greatest reduction in cell death of approximately 68% compared to GFP infected control ($p<0.0005$). Wt-SOD1 infected cells exhibited a dramatic increase in cell death on Hsp infection, as had been observed in the neuronal cell experiments, as well as the rat DRG experiments. On infection of wt-SOD1 cells with Hsp27 death increased sharply by approximately 140% and by approximately 151% on Hsp70 infection, compared to GFP infected control ($p<0.0005$). As with previous experiments in neuronal cells and rat DRGs the dual infection of Hsp27 and Hsp70 of wt-SOD1 cells brought the level of death down toward control GFP levels but still approximately 34% higher than control GFP ($p<0.0005$).

TUNEL analysis provided similar results with G93R mutant SOD infected mouse DRG exhibiting a reduction in the number of TUNEL positive cells by approximately 45% and 63% on infection with Hsp27 and Hsp70 respectively compared to GFP infected control ($p<0.0005$); (Figure 5.12b). On dual infection of Hsp27 and Hsp70 the G93R infected cells exhibited the greatest reduction in the number of TUNEL positive cells with the number decreasing by approximately 71% compared to GFP infected control ($p<0.0005$). Wt-SOD1 infected cells exhibited a dramatic increase in the number of TUNEL positive cells on Hsp infection, with Hsp27 infection bringing about a 172% increase and Hsp70 infection an approximately 185% increase, compared to GFP infected control ($p<0.0005$). The dual infection of Hsp27 and Hsp70 of wt-SOD1 infected DRGs brought the number of TUNEL positive cells down toward control GFP levels but still approximately 65% higher the control GFP ($p<0.0005$).

Figure 5.12a

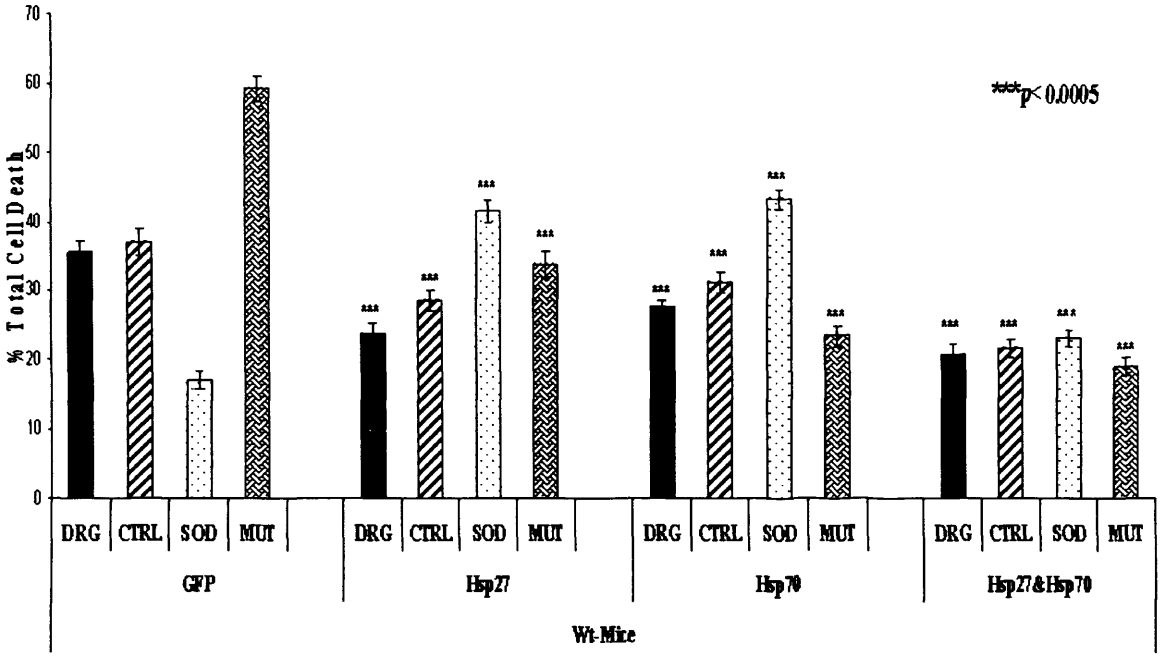



**Figure 5.12 Effect of NGF withdrawal on cell death in control, wild type or mutant SOD1 virus infected wt-mice DRGs**

**a.** Wt-mice DRG cell death following 24h of NGF withdrawal after co-infection with HSV vectors expressing wt-SOD1 or G93R mutant and Hsp27, Hsp70 or Hsp27 and Hsp70. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of treatment of NGF withdrawal of wt-mice DRG cells co-infected with wt or G93R-mutant SOD1 or GFP control and Hsp27, Hsp70 or Hsp27 and Hsp70. Cells were infected with SOD and Hsp viruses 16h prior to stress. Bars represent mean $\pm$ S.D calculated for triplicate counts per sample (n), n=4. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p$<0.0005). *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

Figure 5.12b

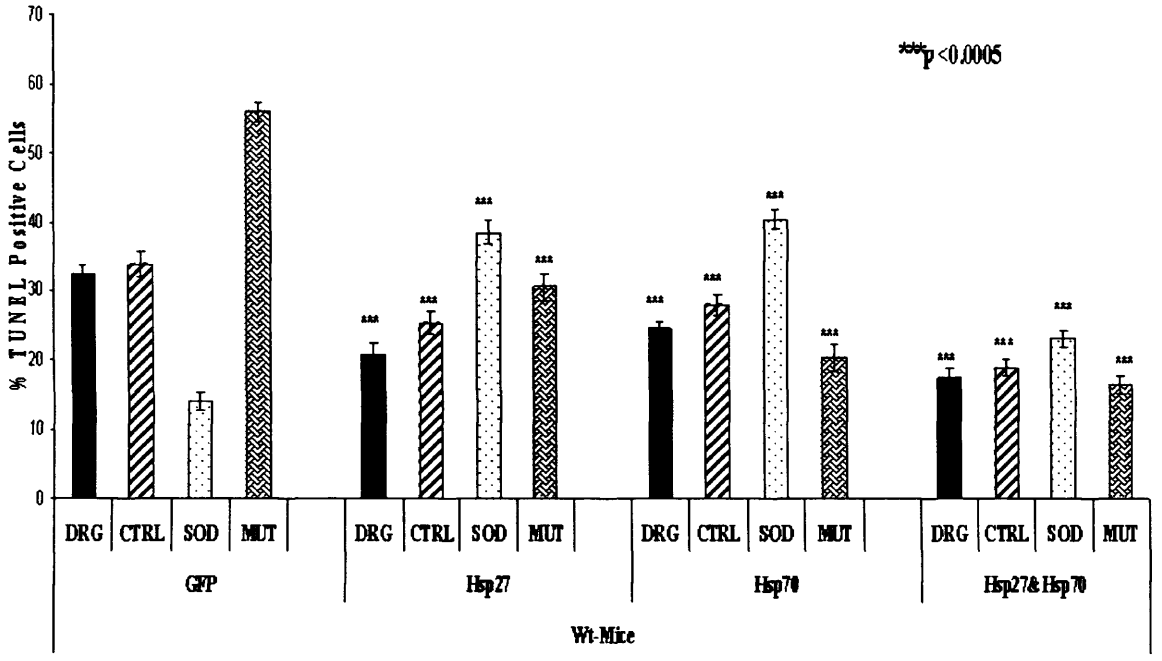



**Figure 5.12 Effect of NGF withdrawal on cell death in control, wild type or mutant SOD1 virus infected wt-mice DRGs**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were co-infected with viruses to over-express wt-SOD1, G93R-mutant or GFP and Hsp27, Hsp70 or Hsp27 and Hsp70 16h prior to treatment of NGF withdrawal for 24h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=4) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

### 5.8.2 IFN-γ administration

On exposure to IFN-γ G93R mutant SOD1 infected mice DRGs exhibited a reduction in cell death on infection with Hsp-expressing viruses. On infection with Hsp27 the G93R mutant cell death was reduced by approximately 42% compared to GFP control ($p<0.0005$); (Figure 5.13a). Hsp70 infection brought a greater reduction in death in the G93R mutant cells with an approximately 62% reduction in death compared to GFP control ($p<0.0005$). The dual infection of Hsp27 and Hsp70 reduced the mutant death even further with death being reduced by approximately 68% compared to GFP infected control ($p<0.0005$). Wt-SOD1 infected cells on infection with Hsp's exhibited an increase in cell death. Wt-SOD1 infected cells infected with Hsp27 saw an increase of approximately 150% and those infected with Hsp70 an increase of approximately 192% compared to GFP infected control ($p<0.0005$). The dual infection of wt-SOD1 cells with Hsp27 and Hsp70 brought the cell death down toward control GFP levels however death was still approximately 52% higher than control GFP ($p<0.0005$).

TUNEL analysis confirmed the findings of the trypan blue assay with G93R mutant SOD1 infected mice DRGs exhibiting a reduction in the number of TUNEL positive cells on infection with Hsps. On infection with Hsp27 the G93R mutant exhibited a reduction in the number of TUNEL positive cells by approximately 44% compared to GFP control ($p<0.0005$); (Figure 5.13b). Hsp70 infection brought a greater reduction in the number of TUNEL positive cells of G93R mutant with the number decreasing by approximately 65% compared to GFP control ($p<0.0005$). The dual infection of the G93R mutant with Hsp27 and Hsp70 reduced the number of TUNEL positive cells even further with the number reduced by approximately 71% compared to GFP infected control ($p<0.0005$). Wt-SOD1 infected cells on infection with Hsps exhibited an increase in cell death. Wt-SOD1 infected cells infected with Hsp27 saw an increase in the number of TUNEL positive cells by approximately 187% and those infected with Hsp70 an increase of approximately 239% compared to GFP infected control ($p<0.0005$). The dual infection of wt-SOD1 cells with Hsp27 and Hsp70 brought the number of TUNEL positive cells down toward control GFP levels however death was still approximately 65% higher than control GFP ($p<0.0005$).

Figure 5.13a

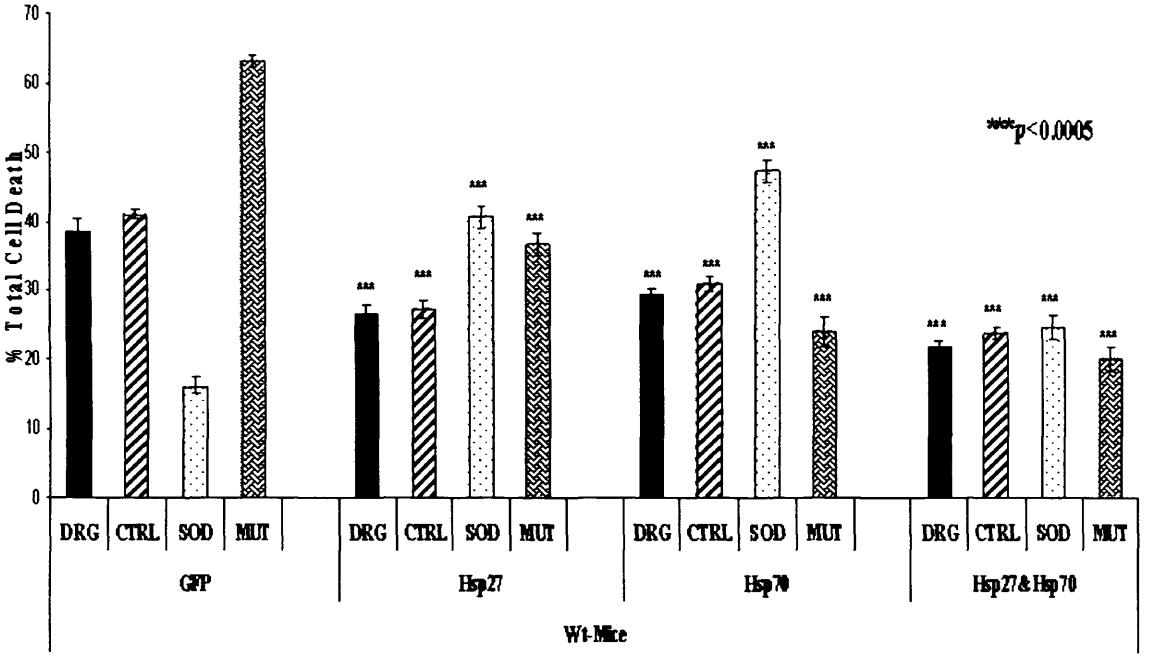



**Figure 5.13 Effect of IFN-γ on cell death in control, wild type or mutant SOD1 virus infected wt-mice DRGs**

**a.** Wt-mice DRG cell death following 24h of treatment with 50ng/ml IFN-γ after infection with HSV vectors expressing wt-SOD1 or G93R mutant and Hsp27, Hsp70 or Hsp27 and Hsp70. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of treatment of 50ng/ml IFN-γ of wt-mice DRG cells infected with wt or G93R-mutant SOD1 or GFP control and Hsp27, Hsp70 or Hsp27 and Hsp70. Cells were co-infected with SOD and Hsp viruses 16h prior to stress. Bars represent mean $\pm$ S.D calculated for triplicate counts per sample (n), n=4. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p<0.0005$). *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

Figure 5.13b

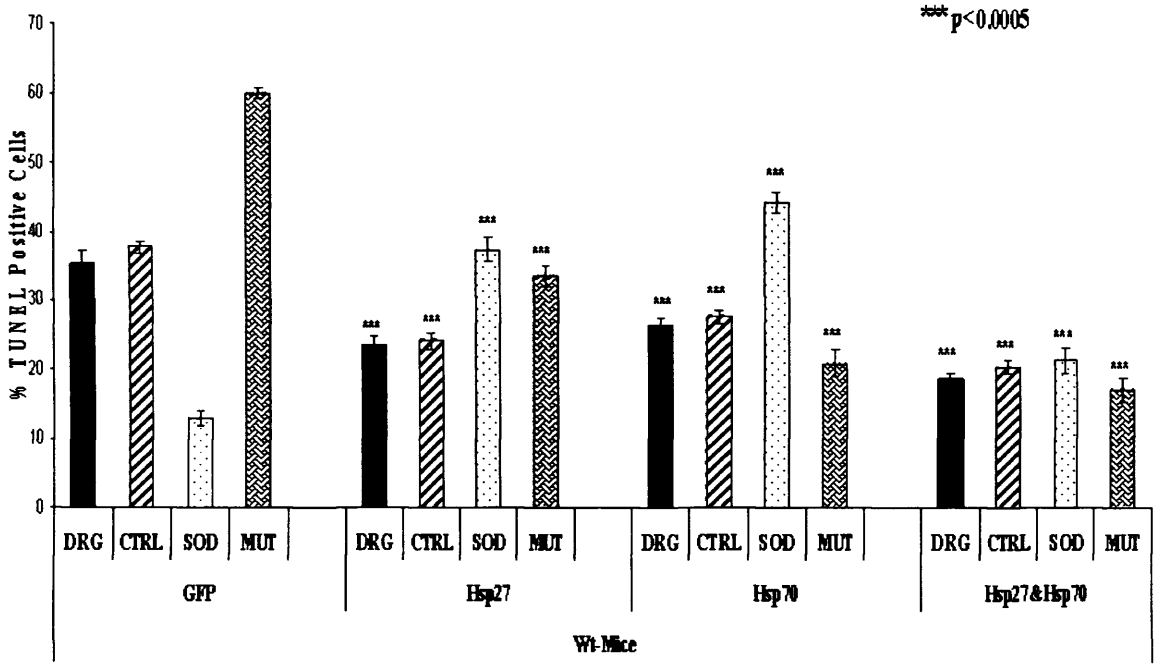



**Figure 5.13 Effect of IFN-γ on cell death in control, wild type or mutant SOD1 virus infected wt-mice DRGs**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were co-infected with viruses to over-express wt-SOD1, G93R-mutant or GFP and Hsp27, Hsp70 or Hsp27 and Hsp70 16h prior to treatment of 50ng/ml IFN-γ for 24h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=4) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

### 5.8.3 Staurosporine treatment

Infection of mutant G93R infected DRGs with Hsp-expressing viruses brought about a reduction in the death of the mutant, as had been observed in the previous experiments. Hsp27 infection of mutant G93R cells reduced death by approximately 68% and Hsp70 infection brought about a 73% reduction compared to GFP infected control ($p<0.0005$); (Figure 5.14a). The dual infection of G93R cells with Hsp27 and Hsp70 further reduced death with a reduction of approximately 80% compared to GFP control ($p<0.0005$). The wt-SOD1 cells once again on infection with Hsp27 or Hsp70 showed a dramatic increase in cell death with death rising by approximately 63% and 139% respectively compared to control GFP ($p<0.0005$). The dual infection of the wt-SOD1 cells with Hsp27 and Hsp70 reduced death down to control GFP levels.

TUNEL analysis provided similar results with infection of mutant G93R DRGs with Hsp's bringing about a reduction in the number of TUNEL positive cells. Hsp27 infection of mutant G93R cells reduced the number of TUNEL positive cells by approximately 71% and Hsp70 infection brought about a 76% reduction compared to GFP infected control ($p<0.0005$); (Figure 5.14b). The dual infection of G93R cells with Hsp27 and Hsp70 further reduced the number of TUNEL positive cells with a reduction of approximately 84% compared to GFP control ($p<0.0005$). The wt-SOD1 cells once again on infection with Hsp27 or Hsp70 showed a dramatic increase in the number of TUNEL positive cells with the number rising by approximately 77% and 168% respectively compared to control GFP ($p<0.0005$). The dual infection of the wt-SOD1 cells with Hsp27 and Hsp70 reduced the number of TUNEL positive cells back down to control GFP levels.

Figure 5.14a

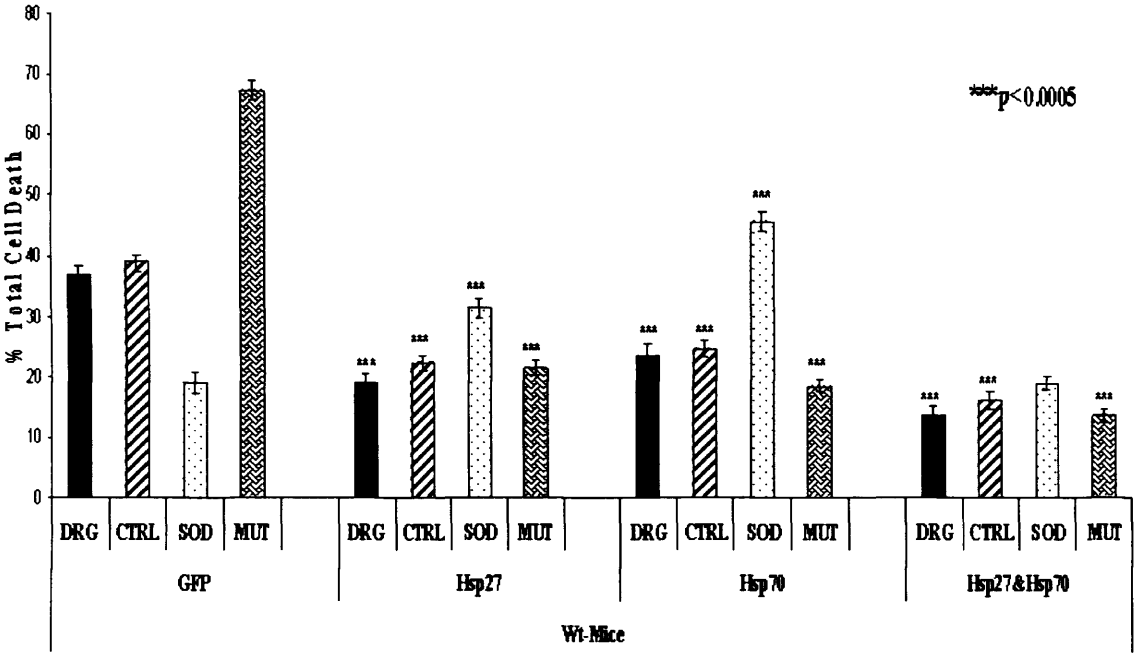



**Figure 5.14 Effect of Staurosporine on cell death in control, wild type or mutant SOD1 virus infected wt-mice DRGs**

**a.** Wt-mice DRG cell death following 24h of 1μM staurosporine administration after co-infection with HSV vectors expressing wt-SOD1 or G93R mutant and Hsp27, Hsp70 or Hsp27 and Hsp70. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of treatment of 1μM staurosporine of wt-mice DRG cells co-infected with wt or G93R-mutant SOD1 or GFP control and Hsp27, Hsp70 or Hsp27 and Hsp70. Cells were co-infected with SOD and Hsp viruses 16h prior to stress. Bars represent mean $\pm$ S.D calculated for triplicate counts per sample (n), n=4. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p<0.0005$). *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

Figure 5.14b

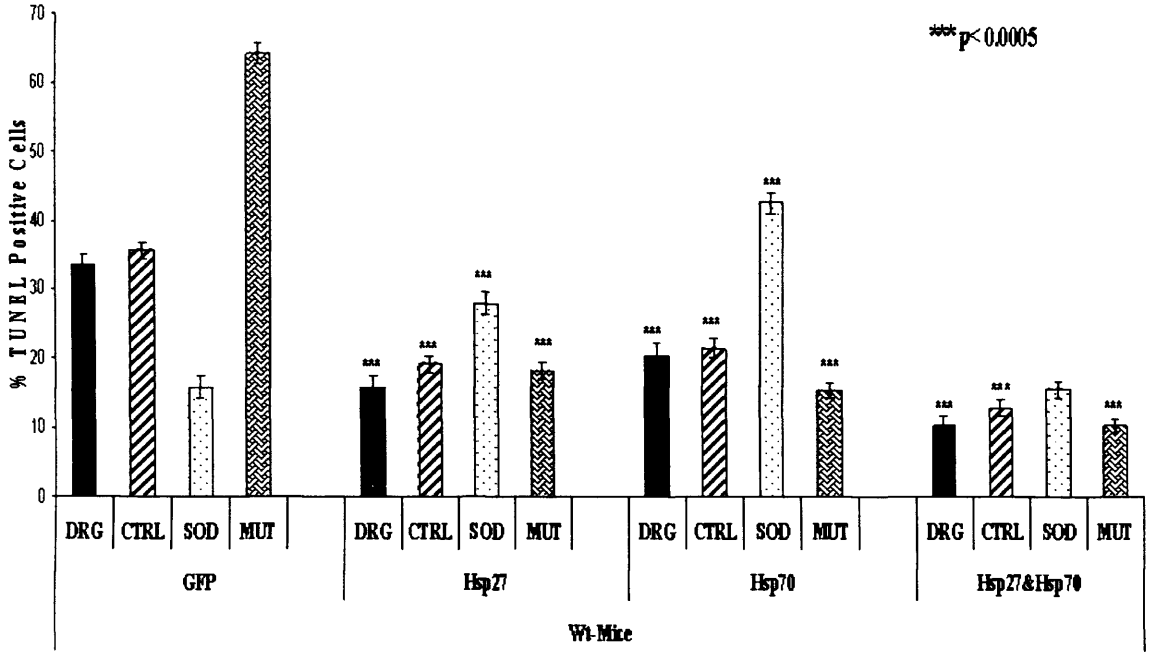



**Figure 5.14 Effect of Staurosporine on cell death in control, wild type or mutant SOD1 virus infected wt-mice DRGs**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were co-infected with viruses to over-express wt-SOD1, G93R-mutant or GFP and Hsp27, Hsp70 or Hsp27 and Hsp70 16h prior to treatment of 1μM staurosporine for 24h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=4) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

## 5.9 Response of Transgenic Hsp27 mice DRGs to stress following over-expression of wt or G93R mutant SOD1

As mentioned previously, the viruses used in the present study, offer a high efficiency means of specific gene delivery to primary neuronal cells (90-100% transduction efficiency). The Hsp viruses used in the present study were constructed in our laboratory by Dr Marcus Wagstaff (Wagstaff et al., 1998). The transgenes (Chinese hamster Hsp27, human inducible Hsp70, or GFP) in this type of virus are driven by the human CMV-IE promoter which was inserted immediately downstream of the LAT P2 promoter. The SOD1 viruses (wt-SOD1, G93R-mutant SOD1 and GFP-control) were constructed in our lab by Dr Yolanda Collaco-Moraes. The pR20.5 cassette consists of a central LAT P2 element flanked by two heterologous promoters (CMV-IE and RSV) arranged in a back-to-back orientation, allowing simultaneous expression of either wt-SOD1 or G93R-SOD1 mutant (under the Rous Sarcoma Virus - RSV- promoter) and GFP (under CMV-IE promoter) and enabling long term expression of both transgenes.

In order to investigate the protective effects of Hsps further, we infected primary cultures of Transgenic Hsp27 (Tg-Hsp27) and Transgenic Hsp70 (Tg-Hsp70) DRGs with wt-SOD1, G93R-SOD1 mutant or control GFP virus. The transgenic Hsp27 (Tg-Hsp27) mice were a gift from Professor J. de Belleroche, Imperial College. The Hsp70 transgenic mice (Tg-Hsp70) were a gift from Dr. C.E. Angelidis, Dalhousie University, Canada. The Hsp27 transgenic mice incorporate a transgene containing human Hsp27 cDNA with a chicken -actin promoter and cytomegalovirus enhancer (pCAGGS) (see Akbar et al., 2003). The Hsp70 transgenic mice incorporate human inducible Hsp70 gene under the regulation of β-actin promoter (see Plumier et al., 1995).

The combined effects of Hsps were investigated further by additional delivery of either Hsp27 to Tg-Hsp70 DRGs or Hsp70 to Tg-Hsp27 DRGs. The primary cultures were then subjected 16h post infection to NGF withdrawal, IFN-γ or staurosporine administration. The findings for the various stresses are presented below the corresponding stress. As with previous experiments, cell death was assessed by trypan blue exclusion assay and TUNEL analysis.

### 5.9.1 NGF withdrawal

Transgenic Hsp27 mice (Tg-Hsp27) DRGs infected with G93R mutant virus exhibited an approximately 63% reduction in death compared to wt-mice DRGs infected with G93R-mutant virus ($p<0.0005$). Wt-SOD1 infection of Tg-Hsp27 DRGs led to an increase in death of approximately 76% compared to wt-mice DRGs infected with wt-SOD1 virus ($p<0.0005$); (Figure 5.15a). Delivery of Hsp70 to G93R-mutant infected Tg-Hsp27 DRGs led to a 57% ($p<0.0005$) reduction in death compared to G93R-mutant infected Tg-Hsp27 DRGs and approximately 84% ($p<0.0005$) reduction in death compared to G93R-mutant infected wt-mice DRGs. Hsp70 delivery to wt-SOD1 infected Tg-Hsp27 DRGs led to a 55% ($p<0.0005$) decrease in cell death compared to wt-SOD1 infected Tg-Hsp27 DRGs and approximately 20% ($p<0.0005$) reduction in total cell death compared to wt-SOD1 infected wt-mice DRGs.

TUNEL analysis confirmed these findings with Tg-Hsp27 mice DRGs infected with G93R mutant virus exhibiting an approximately 65% reduction in the number of TUNEL positive cells compared to G93R-SOD1 mutant infected wt-mice DRGs ($p<0.0005$). Wt-SOD1 infection of Tg-Hsp27 DRGs led to an increase in the number of TUNEL positive cells by approximately 102% compared to wt-SOD1 virus infected wt-mice DRGs ($p<0.0005$); (Figure 5.15b). Delivery of Hsp70 to G93R-mutant infected Tg-Hsp27 DRGs led to a 64% ($p<0.0005$) reduction in the number of TUNEL positive cells compared to G93R-mutant infected Tg-Hsp27 DRGs and approximately 87% ($p<0.0005$) reduction in the number of TUNEL positive cells compared to G93R-SOD1 mutant infected wt-mice DRGs. Delivery of Hsp70 to wt-SOD1 infected Tg-Hsp27 DRGs led to a 59% ($p<0.0005$) decrease in the number of TUNEL positive cells compared to wt-SOD1 infected Tg-Hsp27 DRGs and approximately 17% ($p<0.0005$) reduction in the number of TUNEL positive cells compared to wt-SOD1 infected wt-mice DRGs.

Figure 5.15a

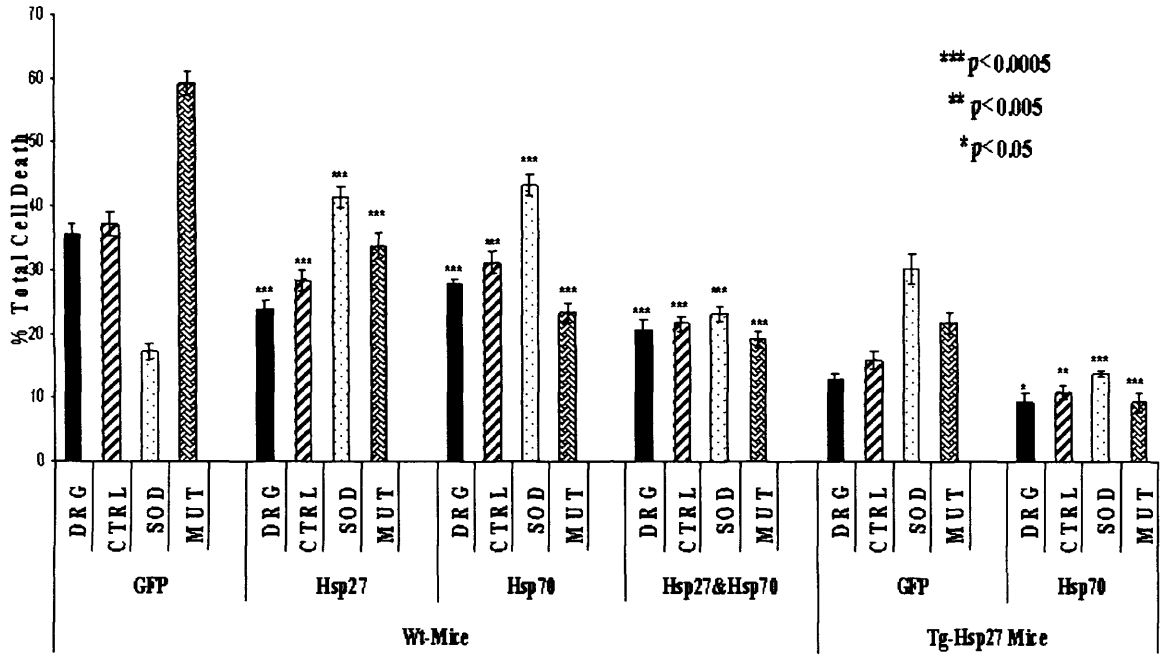



**Figure 5.15 Effect of NGF withdrawal on cell death in control, wild type or mutant SOD1 virus infected wt-mice DRGs and Tg-Hsp27 DRGs**

**a.** Wt and Tg-Hsp27 mice DRG cell death following 24h of NGF withdrawal after co-infection with HSV vectors expressing wt-SOD1 or G93R mutant and Hsp27, Hsp70 or Hsp27 and Hsp70. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of treatment of NGF withdrawal of wt-mice and Tg-Hsp27 DRG cells co-infected with wt or G93R-mutant SOD1 or GFP control and Hsp27, Hsp70 or Hsp27 and Hsp70. Cells were infected with SOD and Hsp viruses 16h prior to stress. Bars represent mean $\pm$ S.D calculated for triplicate counts per sample (n), n=4. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p<0.0005$). *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

Figure 5.15b

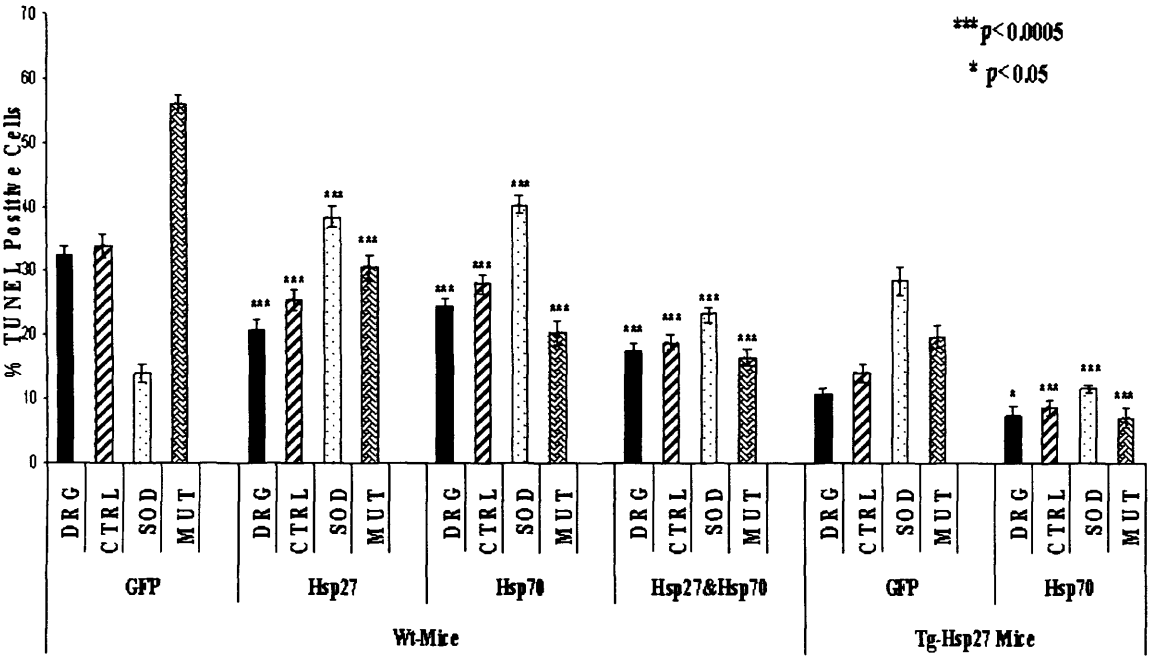



**Figure 5.15 Effect of NGF withdrawal on cell death in control, wild type or mutant SOD1 virus infected wt-mice DRGs and Tg-Hsp27 DRGs**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were co-infected with viruses to over-express wt-SOD1, G93R-mutant or GFP and Hsp27, Hsp70 or Hsp27 and Hsp70 16h prior to treatment of NGF withdrawal for 24h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=4) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

### 5.9.2 IFN-γ administration

Tg-Hsp27 DRGs infected with G93R mutant virus exhibited a 61% lower death than G93R mutant infected wt-mice DRGs ($p<0.0005$); (Figure 5.16a). Wt-SOD1 infected Tg-Hsp27 DRGs exhibited a 61% higher death compared to wt-SOD1 infected wt-mice DRGs ($p<0.0005$). Delivery of Hsp70 to G93R infected Tg-Hsp27 DRGs reduced death by approximately 57% ($p<0.0005$) compared to G93R mutant infected Tg-Hsp27 cells and approximately 83% ($p<0.0005$) compared to G93R-mutant infected wt-mice DRGs. Wt-SOD1 infected Tg-Hsp27 cells saw an approximately 43% ($p<0.0005$) reduction in cell death on delivery of Hsp70 compared to wt-SOD1 infected Tg-Hsp27 DRGs.

TUNEL analysis revealed similar results with Tg-Hsp27 DRGs infected with G93R mutant virus exhibiting a 62% reduction in the number of TUNEL positive cells compared to G93R mutant infected wt-mice DRGs ($p<0.0005$) (Figure 5.16b). Wt-SOD1 infected Tg-Hsp27 DRGs exhibited a 96% increase in the number of TUNEL positive cells compared to wt-SOD1 infected wt-mice DRGs ($p<0.0005$). Delivery of Hsp70 to G93R mutant infected Tg-Hsp27 DRGs reduced the number of TUNEL positive cells by approximately 63% ($p<0.0005$) compared to G93R mutant infected Tg-Hsp27 cells and approximately 86% ($p<0.0005$) compared to G93R-mutant infected wt-mice DRGs. Delivery of Hsp70 to wt-SOD1 infected Tg-Hsp27 cells brought about a reduction in the number of TUNEL positive cells by approximately 49% ($p<0.0005$) compared to wt-SOD1 infected Tg-Hsp27.

Figure 5.16a

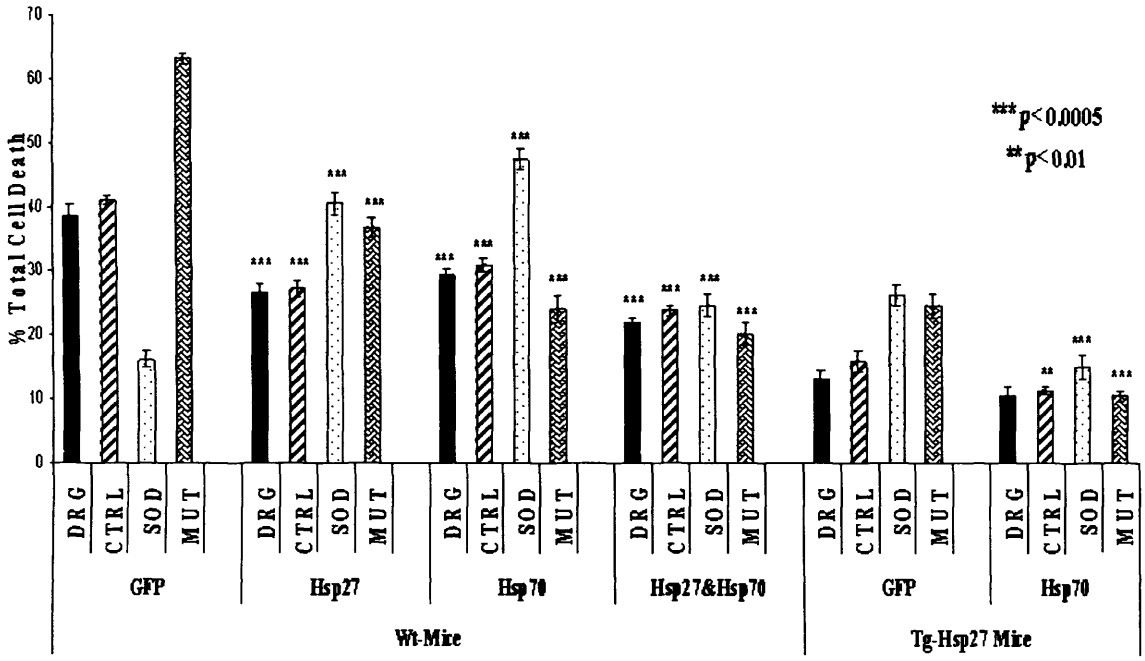



**Figure 5.16 Effect of IFN-γ on cell death in control, wild type or mutant SOD1 virus infected wt-mice DRGs and Tg-Hsp27 DRGs**

**a.** Wt-mice and Tg-Hsp27 DRG cell death following 24h of treatment with 50ng/ml IFN-γ after infection with HSV vectors expressing wt-SOD1 or G93R mutant and Hsp27, Hsp70 or Hsp27 and Hsp70. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of treatment of 50ng/ml IFN-γ of wt-mice and Tg-Hsp27 DRG cells infected with wt or G93R-mutant SOD1 or GFP control and Hsp27, Hsp70 or Hsp27 and Hsp70. Cells were co-infected with SOD and Hsp viruses 16h prior to stress. Bars represent mean $\pm$ S.D calculated for triplicate counts per sample (n), n=4. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p<0.0005$). *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

Figure 5.16b

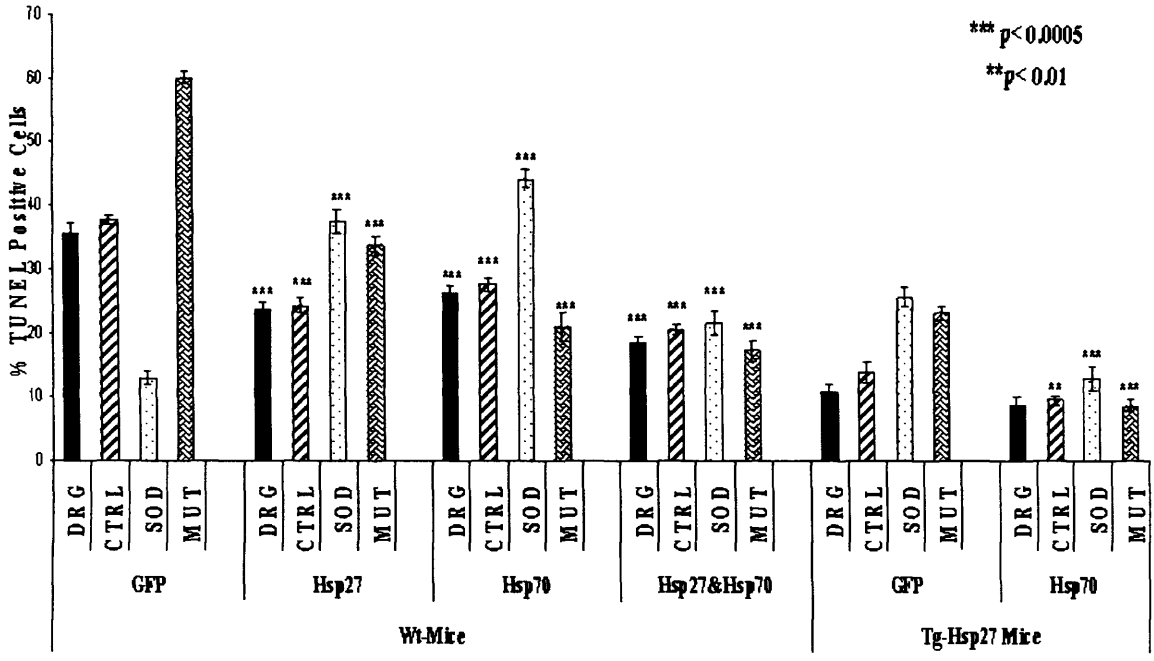



**Figure 5.16 Effect of IFN-γ on cell death in control, wild type or mutant SOD1 virus infected wt-mice DRGs and Tg-Hsp27 DRGs**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were co-infected with viruses to over-express wt-SOD1, G93R-mutant or GFP and Hsp27, Hsp70 or Hsp27 and Hsp70 16h prior to treatment of 50ng/ml IFN-γ for 24h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=4) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

### 5.9.3 Staurosporine treatment

G93R mutant infected Tg-Hsp27 DRGs showed an approximately 48% reduction in death compared to G93R mutant infected wt-mice DRGs ($p<0.0005$). Wt-SOD1 infected Tg-Hsp27 DRGs showed an increase in death of approximately 199% compared to wt-SOD1 infected wt-mice DRGs ($p<0.0005$); (Figure 5.17a). Delivery of Hsp70 to G93R mutant infected Tg-Hsp27 cells led to a reduction in death of approximately 55% ($p<0.0005$) compared to G93R infected Tg-Hsp27 cells and approximately 76% ($p<0.0005$) reduction in total cell death compared to G93R-mutant infected wt-mice DRGs. Delivery of Hsp70 to wt-SOD1 infected Tg-Hsp27 cells reduced death by approximately 63% compared to wt-SOD1 infected Tg-Hsp27 DRGs ($p<0.0005$).

TUNEL analysis revealed similar results with G93R mutant infected Tg-Hsp27 DRGs exhibiting a reduction in the number of TUNEL positive cells by approximately 48% compared to G93R mutant infected wt-mice DRGs ($p<0.0005$). Wt-SOD1 infected Tg-Hsp27 DRGs showed an increase in the number of TUNEL positive cells by approximately 199% compared to wt-SOD1 infected wt-mice DRGs ($p<0.0005$); (Figure 5.17b). Delivery of Hsp70 to G93R mutant infected Tg-Hsp27 cells led to a reduction of approximately 58% ($p<0.0005$) in the number of TUNEL positive cells compared to G93R infected Tg-Hsp27 cells and approximately 78% ($p<0.0005$) reduction in the number of TUNEL positive cells compared to G93R-mutant infected wt-mice DRGs. Delivery of Hsp70 to wt-SOD1 infected Tg-Hsp27 brought about an approximately 65% a reduction in number of TUNEL positive cells compared to wt-SOD1 infected Tg-Hsp27 DRGs ($p<0.0005$).

Figure 5.17a

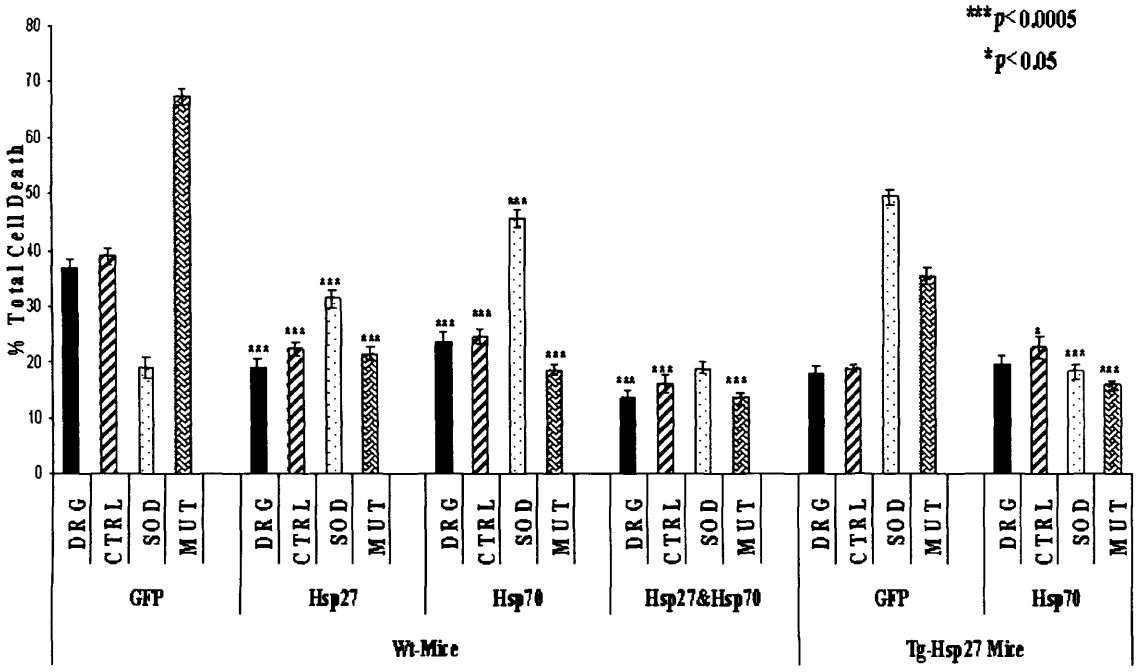



**Figure 5.17 Effect of Staurosporine on cell death in control, wild type or mutant SOD1 virus infected wt-mice DRGs and Tg-Hsp27 DRGs**

**a.** Wt-mice and Tg-Hsp27 DRG cell death following 24h of 1μM staurosporine administration after co-infection with HSV vectors expressing wt-SOD1 or G93R mutant and Hsp27, Hsp70 or Hsp27 and Hsp70. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of treatment of 1μM staurosporine of wt-mice and Tg-Hsp27 DRG cells co-infected with wt or G93R-mutant SOD1 or GFP control and Hsp27, Hsp70 or Hsp27 and Hsp70. Cells were co-infected with SOD and Hsp viruses 16h prior to stress. Bars represent mean $\pm$ S.D calculated for triplicate counts per sample (n), n=4. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p<0.0005$). *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

Figure 5.17b

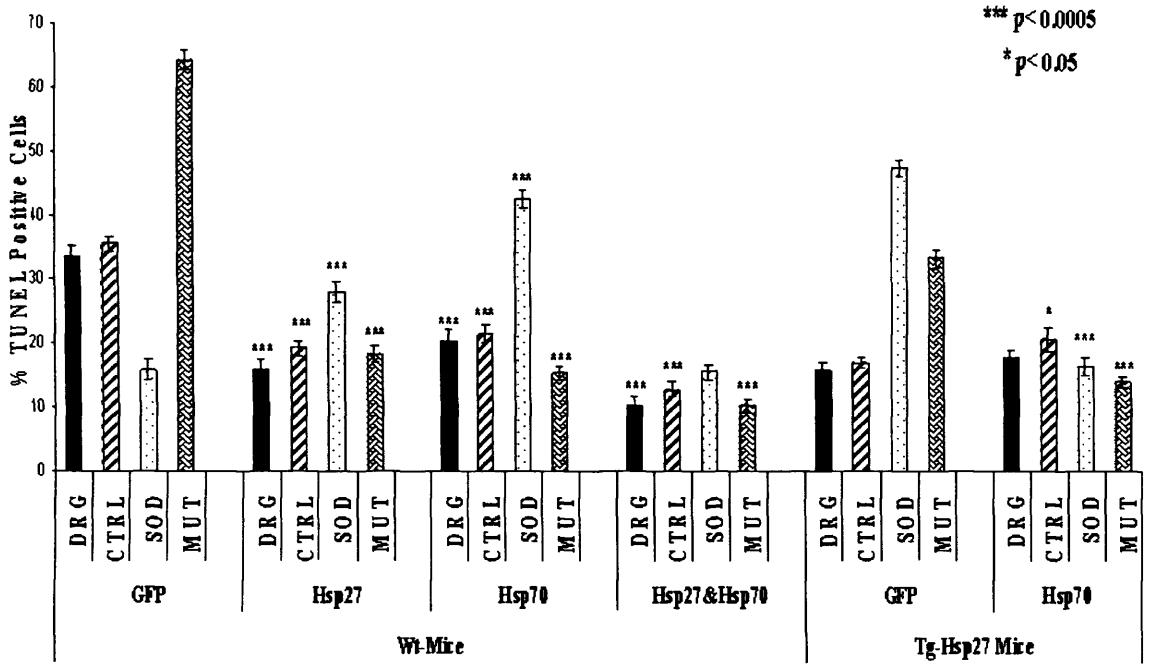



**Figure 5.17 Effect of Staurosporine on cell death in control, wild type or mutant SOD1 virus infected wt-mice DRGs and Tg-Hsp27 DRGs**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were co-infected with viruses to over-express wt-SOD1, G93R-mutant or GFP and Hsp27, Hsp70 or Hsp27 and Hsp70 16h prior to treatment of 1μM staurosporine for 24h. Bars represent mean $^{\pm}$ S.D. calculated for triplicate counts per sample (n=4) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

## 5.10 Response of Transgenic Hsp70 mice DRGs to stress following over-expression of wt or G93R mutant SOD1

### 5.10.1 NGF withdrawal

G93R infected Tg-Hsp70 mice DRGs exhibited an approximately 73% reduction in death compared to wt-mice DRGs infected with G93R mutant virus ($p<0.0005$). Wt-SOD1 infected Tg-Hsp70 DRGs exhibited a 248% increase in death compared to wt-mice DRGs infected with wt-SOD1 virus ($p<0.0005$); (Figure 5.18a). Delivery of Hsp27 to G93R mutant infected Tg-Hsp70 DRGs reduced death by approximately 27% ($p<0.005$) compared to G93R mutant Tg-Hsp70 DRGs and approximately 80% ($p<0.0005$) compared to G93R-mutant infected wt-mice DRGs. Wt-SOD1 infected Tg-Hsp70 DRGs saw a reduction in death by approximately 66% on delivery of Hsp27 compared to wt-SOD1 infected Tg-Hsp70 DRGs ($p<0.0005$).

TUNEL analysis confirmed the findings on the trypan blue analysis with G93R infected Tg-Hsp70 mice DRGs exhibiting an approximately 75% reduction in the number of TUNEL positive cells compared to wt-mice DRGs infected with G93R mutant virus($p<0.0005$). Wt-SOD1 infected Tg-Hsp70 DRGs exhibited a 311% increase in the number of TUNEL positive cells compared to wt-mice DRGs infected with wt-SOD1 virus ($p<0.0005$); (Figure 5.18b). Delivery of Hsp27 to G93R mutant infected Tg-Hsp70 DRGs reduced the number of TUNEL positive cells by approximately 30% ($p<0.005$) compared to G93R mutant Tg-Hsp70 DRGs and approximately 83% ($p<0.0005$) compared to G93R-mutant infected wt-mice DRGs. Wt-SOD1 infected Tg-Hsp70 DRGs saw a reduction in the number of TUNEL positive cells by approximately 68% ($p<0.0005$) following delivery of Hsp27 compared to wt-SOD1 infected Tg-Hsp70 DRGs.

Figure 5.18a

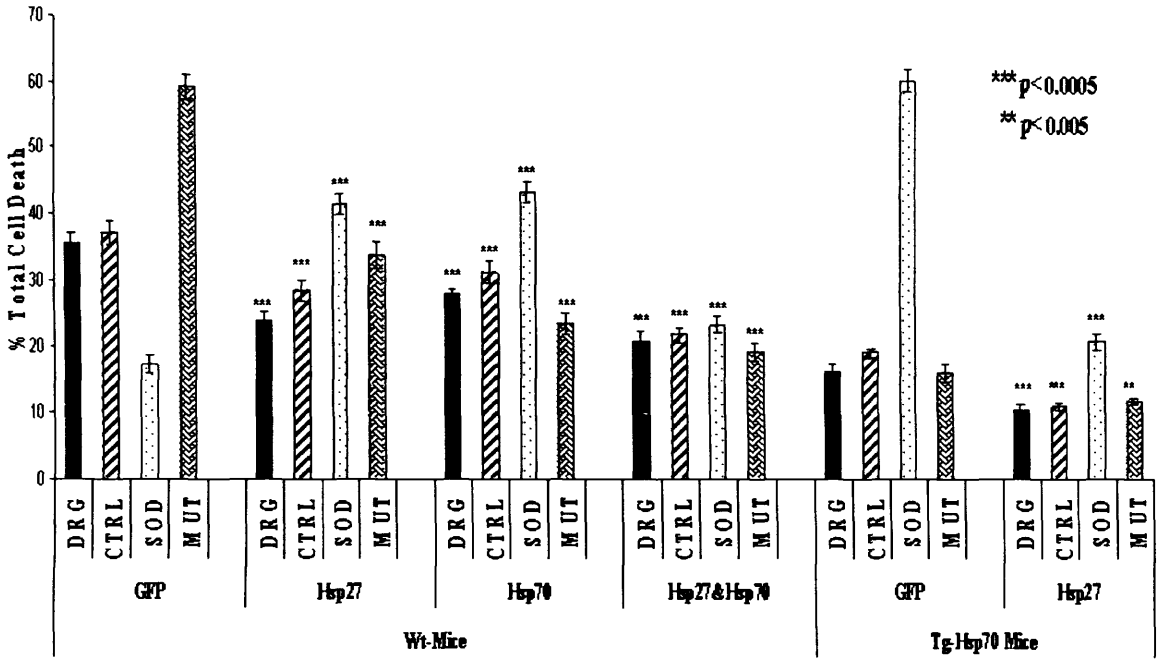



**Figure 5.18 Effect of NGF withdrawal on cell death in control, wild type or mutant SOD1 virus infected wt-mice DRGs and Tg-Hsp70 DRGs**

**a.** Wt and Tg-Hsp70 mice DRG cell death following 24h of NGF withdrawal after co-infection with HSV vectors expressing wt-SOD1 or G93R mutant and Hsp27, Hsp70 or Hsp27 and Hsp70. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of treatment of NGF withdrawal of wt-mice and Tg-Hsp70 DRG cells co-infected with wt or G93R-mutant SOD1 or GFP control and Hsp27, Hsp70 or Hsp27 and Hsp70. Cells were infected with SOD and Hsp viruses 16h prior to stress. Bars represent mean $\pm$ S.D calculated for triplicate counts per sample (n), n=4. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p<0.0005$). *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

Figure 5.18b

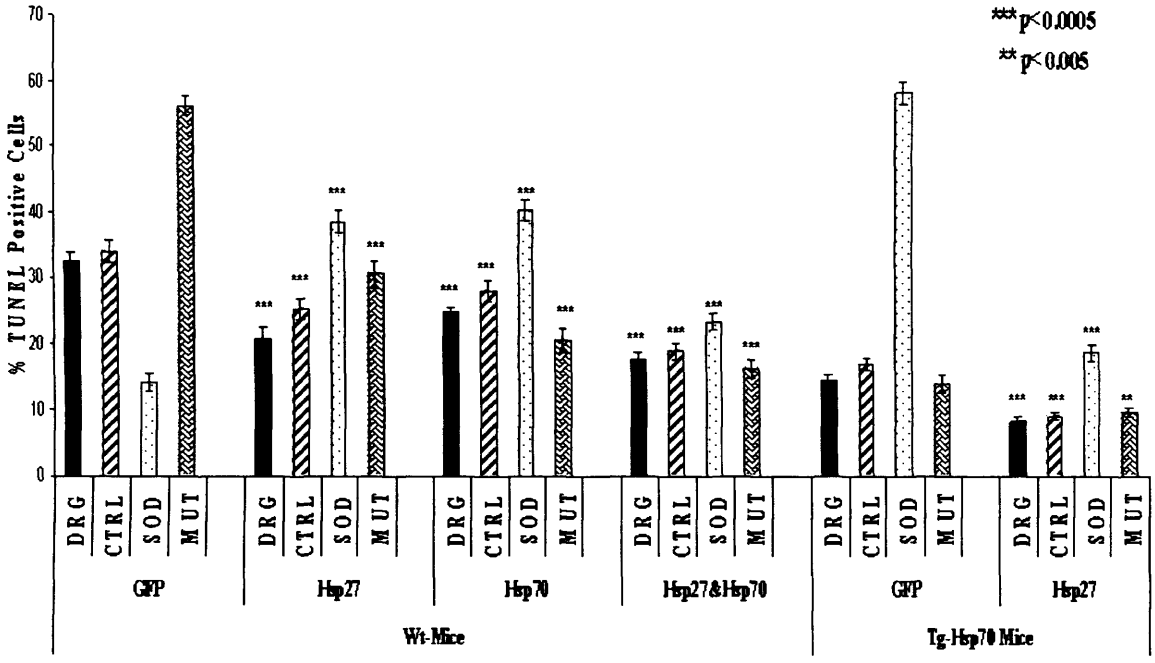



**Figure 5.18 Effect of NGF withdrawal on cell death in control, wild type or mutant SOD1 virus infected wt-mice DRGs and Tg-Hsp70 DRGs**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were co-infected with viruses to over-express wt-SOD1, G93R-mutant or GFP and Hsp27, Hsp70 or Hsp27 and Hsp70 16h prior to treatment of NGF withdrawal for 24h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=4) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

### 5.10.2 IFN-γ administration

G93R mutant infected Tg-Hsp70 DRGs exhibited a reduction in death of approximately 74% compared to G93R mutant infected wt-mice DRGs ($p<0.0005$). On infection with wt-SOD1, Tg-Hsp70 DRGs saw an increase in death of approximately 198% compared to wt-SOD1 infected wt-mice DRGs ($p<0.0005$); (Figure 5.19a). Delivery of Hsp27 to G93R mutant infected Tg-Hsp70 DRGs led to a reduction in death by approximately 33% ($p<0.0005$) compared to G93R mutant infected Tg-Hsp70 DRGs and an approximately 83% ($p<0.0005$) reduction in total cell death compared to G93R-mutant infected wt-mice DRGs. The death of wt-SOD1 infected Tg-Hsp70 DRGs was reduced by approximately 73% ($p<0.0005$) following delivery of Hsp27 compared to wt-SOD1 infected Tg-Hsp70 DRGs and 19% ($p<0.0005$) compared to wt-SOD1 infected wt-mice DRGs.

TUNEL analysis provided similar results with G93R mutant infected Tg-Hsp70 DRGs exhibiting a reduction in the number of TUNEL positive cells by approximately 76% compared to G93R mutant infected wt-mice DRGs($p<0.0005$). On infection with wt-SOD1, Tg-Hsp70 DRGs saw an increase in the number of TUNEL positive cells by approximately 255% compared to wt-mice DRGs infected with wt-SOD1 ($p<0.0005$); (Figure 5.19b). Delivery of Hsp27 to G93R mutant infected Tg-Hsp70 DRGs led to a reduction in the number of TUNEL positive cells by approximately 37% ($p<0.0005$) compared to G93R mutant infected Tg-Hsp70 DRGs and approximately 85% ($p<0.0005$) compared to G93R-mutant infected wt-mice DRGs. Delivery of Hsp27 to wt-SOD1 infected Tg-Hsp70 DRGs led to a reduction in the number of TUNEL positive cells by approximately 76% ($p<0.0005$) compared to wt-SOD1 infected Tg-Hsp70 DRGs and 15% ($p<0.0005$) compared to wt-SOD1 infected wt-mice DRGs.

Figure 5.19a

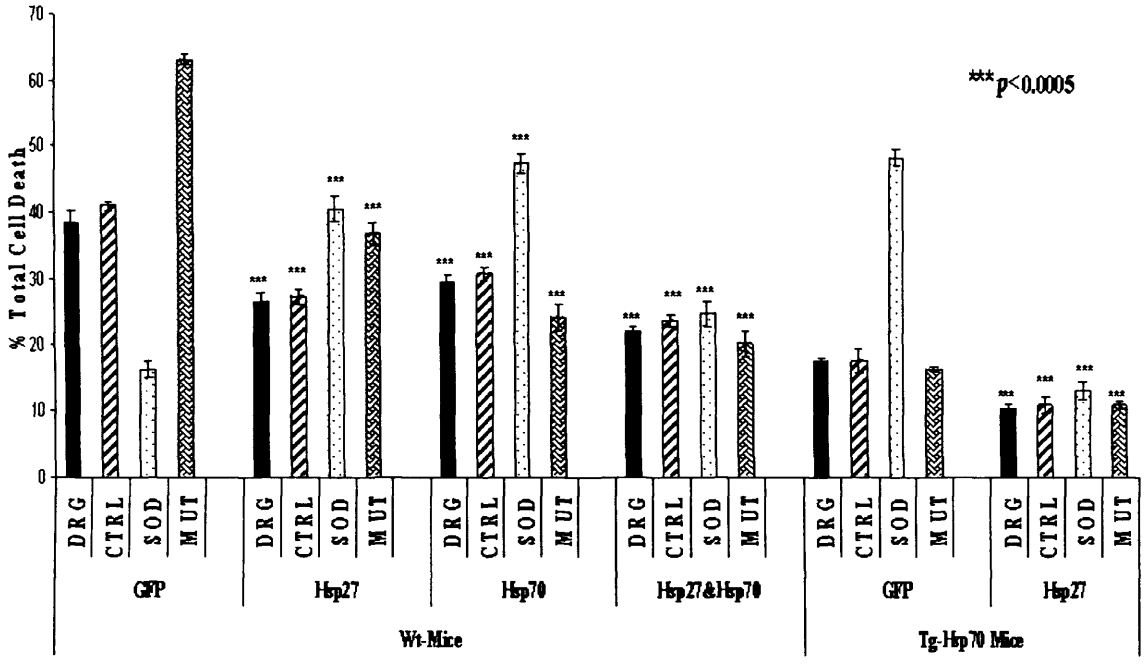



**Figure 5.19 Effect of IFN-γ on cell death in control, wild type or mutant SOD1 virus infected wt-mice DRGs and Tg-Hsp70 DRGs**

**a.** Wt-mice and Tg-Hsp70 DRG cell death following 24h of treatment with 50ng/ml IFN-γ after infection with HSV vectors expressing wt-SOD1 or G93R mutant and Hsp27, Hsp70 or Hsp27 and Hsp70. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of treatment of 50ng/ml IFN-γ of wt-mice and Tg-Hsp70 DRG cells infected with wt or G93R-mutant SOD1 or GFP control and Hsp27, Hsp70 or Hsp27 and Hsp70. Cells were co-infected with SOD and Hsp viruses 16h prior to stress. Bars represent mean $\pm$ S.D calculated for triplicate counts per sample (n), n=4. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p<0.0005$). *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

Figure 5.19b

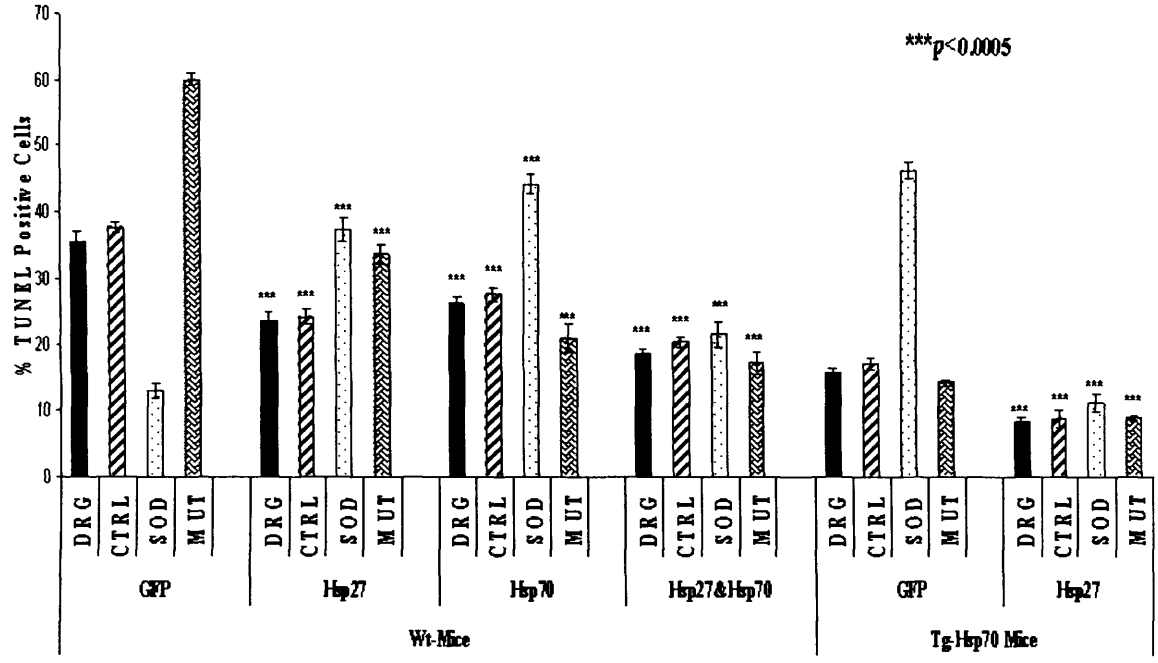



**Figure 5.19 Effect of IFN-γ on cell death in control, wild type or mutant SOD1 virus infected wt-mice DRGs and Tg-Hsp70 DRGs**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were co-infected with viruses to over-express wt-SOD1, G93R-mutant or GFP and Hsp27, Hsp70 or Hsp27 and Hsp70 16h prior to treatment of 50ng/ml IFN-γ for 24h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=4) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

### 5.10.3 Staurosporine treatment

Tg-Hsp70 mutant-G93R infected DRGs exhibited a reduction in death by approximately 68% compared to G93R mutant infected wt-mice DRGs ($p<0.0005$). On infection of Tg-Hsp70 DRGs with wt-SOD1 virus an increase in death of approximately 260% was observed compared to wt-SOD1 infected wt-mice DRGs($p<0.0005$); (Figure 5.20a). Delivery of Hsp27 to G93R mutant infected Tg-Hsp70 DRGs led to a reduction in death by approximately 47% ($p<0.0005$) compared to Tg-Hsp70 DRGs infected with G93R mutant and approximately 83% ($p<0.0005$) reduction in cell death compared to G93R-mutant infected wt-mice DRGs. Wt-SOD1 infected Tg-Hsp70 DRGs showed a reduction in death by approximately 73% on delivery of Hsp27 compared to wt-SOD1 infected Tg-Hsp70 DRGs ($p<0.0005$).

TUNEL analysis provided similar results and confirmed the findings of the trypan blue assay with Tg-Hsp70 mutant-G93R infected DRGs exhibiting a reduction in the number of TUNEL positive cells by approximately 70% compared to G93R mutant infected wt-mice DRGs($p<0.0005$). Infection of Tg-Hsp70 DRGs with wt-SOD1 virus, led to an increase in the number of TUNEL positive cells by approximately 325% compared to wt-SOD1 infected wt-mice DRGs($p<0.0005$); (Figure 5.20b). Delivery of Hsp27 to G93R mutant infected Tg-Hsp70 DRGs, led to a reduction in the number of TUNEL positive cells by approximately 53% ($p<0.0005$) compared to Tg-Hsp70 DRGs infected with G93R mutant and approximately 86% ($p<0.0005$) reduction in cell death compared to G93R-mutant infected wt-mice DRGs. Wt-SOD1 infected Tg-Hsp70 DRGs showed a reduction of approximately 75% in the number of TUNEL positive cells following delivery of Hsp27 compared to wt-SOD1 infected Tg-Hsp70 DRGs($p<0.0005$).

Figure 5.20a

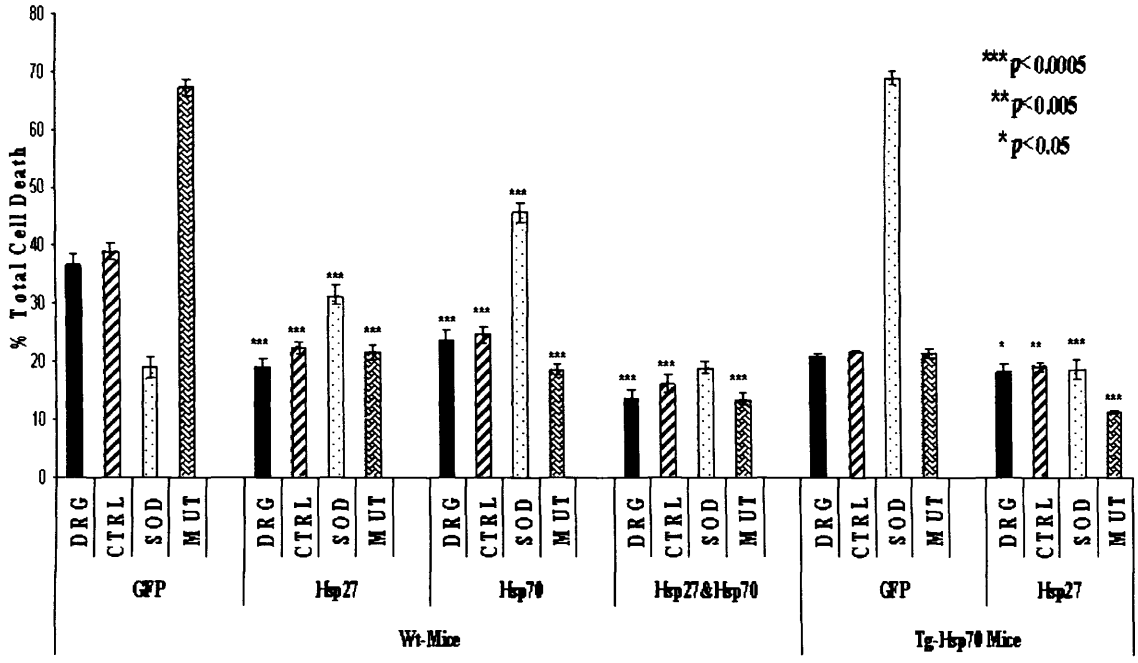



**Figure 5.20 Effect of Staurosporine on cell death in control, wild type or mutant SOD1 virus infected wt-mice DRGs and Tg-Hsp70 DRGs**

**a.** Wt-mice and Tg-Hsp70 DRG cell death following 24h of 1μM staurosporine administration after co-infection with HSV vectors expressing wt-SOD1 or G93R mutant and Hsp27, Hsp70 or Hsp27 and Hsp70. The proportion of cell death was assessed by trypan blue exclusion assay, after 24h of treatment of 1μM staurosporine of wt-mice and Tg-Hsp70 DRG cells co-infected with wt or G93R-mutant SOD1 or GFP control and Hsp27, Hsp70 or Hsp27 and Hsp70. Cells were co-infected with SOD and Hsp viruses 16h prior to stress. Bars represent mean $^{\pm}$S.D. calculated for triplicate counts per sample (n), n=4. Significant differences were calculated using Bonferroni Multiple Comparison's t test after one-way ANOVA ($p<0.0005$). *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

Figure 5.20b

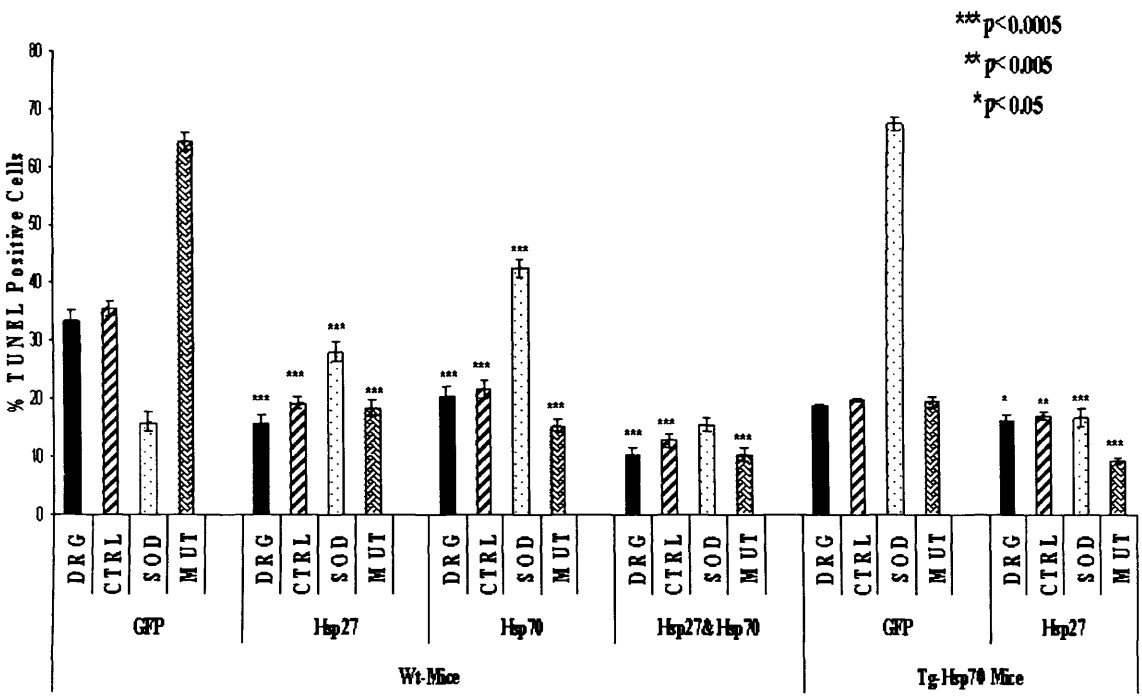



**Figure 5.20 Effect of Staurosporine on cell death in control, wild type or mutant SOD1 virus infected wt-mice DRGs and Tg-Hsp70 DRGs**

**b.** Percentage of TUNEL-positive cells counted in three different fields in three experiments where cell lines were co-infected with viruses to over-express wt-SOD1, G93R-mutant or GFP and Hsp27, Hsp70 or Hsp27 and Hsp70 16h prior to treatment of 1μM staurosporine for 24h. Bars represent mean $\pm$ S.D. calculated for triplicate counts per sample (n=4) and significant differences were calculated as above with $p<0.0005$. *, **, *** indicates statistically significant difference between means, when comparing with GFP virus infected control.

## 5.11 Discussion

This chapter describes the protective effect of heat shock proteins in primary DRG cells against the toxic effects of mutant-SOD1 and provides further support to the findings described in Chapter 4. HSV-based viral vectors were utilised in order to efficiently express Hsp27, Hsp70 or the Hsp27 and Hsp70 combination along with either wt-SOD1 or G93R-mutant. In summary, the wt-SOD1, G93R-mutant and empty vector control infected rat DRGs were infected with the Hsp virus or the relevant control virus and subsequently subjected to stresses including NGF withdrawal, IFN-γ and staurosporine. In addition transgenic Hsp27 or Hsp70 mice and control mice were infected with wt-SOD1, G93R mutant or control virus to further confirm the findings of chapter 4 and those of the rat DRGs. The response of the primary cells to the above death inducing stimuli was quantified through TUNEL analysis to establish the number of apoptotic cells.

The results of chapter 4 indicate that the exogenous expression of Hsp27 or Hsp70 in the *in vitro* mammalian model system presented in this thesis, over-expressing G93A or G93R SOD1 mutants, confer protection against a wide range of lethal stimuli. Briefly, the results presented in this chapter provide further support to the findings of chapter 4 through investigation of protective effects of Hsps in both rat and mouse DRGs. Exogenous expression of Hsp27 and/or Hsp70 confers protection against G93R-mutant induced toxicity in rat and mouse DRGs subjected to a range of lethal stimuli. Wt-SOD1 infected DRGs exhibited increased levels of death on Hsp70 infection bringing about a larger increase in death than Hsp27 as seen in the ND7 cell experiments with wt-SOD1 cells. Hsp70 was much more effective in rescuing the G93R mutant than Hsp27 paralleling the findings of chapter 4. Furthermore, a joint infection of Hsp27 and Hsp70 rescued the G93R SOD1 mutant more effectively than single viral infection with Hsp27 or Hsp70. In addition, as with the findings of chapter 4, the enhanced cell death observed in the wt-SOD1 infected cells on infection with Hsp27 or Hsp70 was abolished on infection with the Hsp27 and Hsp70 combination suggesting that Hsp27 and Hsp70 may act in synergy.

Using retroviral vectors, it was previously shown that Hsp70 overexpression protects astrocytes from glucose deprivation, combined oxygen-glucose deprivation, as well as from $H_2O_2$

exposure (Papadopoulos et al., 1996; Xu and Giffard, 1997). In addition, overexpression of Hsp70 in cultured neurons is also associated with protection (Amin et al., 1996; Beaucamp et al., 1998; Fink et al., 1997; Uney et al, 1993), and overexpression of Hsp70 in astrocytes was found to protect co-cultured wild type neurons (Xu et al., 1999). These *in vitro* injury models mimic to some extent the injury involved in damage brought about by mutant SOD1 toxicity and suggest several ways in which Hsp70 could provide protection. Astrocytes protected from injury by Hsp70 had higher levels of glutathione than control cells under the same conditions of stress (Xu and Giffard, 1997). The ability to refold proteins or prevent aggregation may enable the cell to conserve glutathione and perhaps even ATP (Wong et al., 1998). Studies carried out in both *in vitro* and *in vivo* by Kelly et al. (2002) found that neurons overexpressing Hsp70 were also found to overexpress the anti-apoptotic protein bcl-2.

Despite intensive investigations, the prominent pathogenic mechanisms have as yet not been identified for any type of ALS. It is known from transgenic rodents that high levels of mutant SOD-1 protein kill motor neurons in a dose-dependent manner. In these models, multiple processes play a role in the demise of motor neurons, including early misfolding and aggregation of mutant SOD-1 protein (Bruijn et al., 1997), excitotoxicity mediated by failing synaptic transport of glutamate into glial cells, diminished energy generation as a result of mitochondrial dysfunction, and impaired axonal transport.

In both rodents and humans, distressed motor neurons trigger proliferation of surrounding astrocytes and microglial cells in affected spinal gray matter. Gene expression and biochemical studies reveal characteristics of inflammation, such as cyclooxygenase activation. These findings are apparent in both sporadic ALS cases as well as those associated with mutant SOD-1. The process of motor neuron death is not cell-autonomous but rather it is considered to be intimately related to the context of surrounding cells. Clement et al. (2003) reported cell death of motor neurons expressing mutant SOD-1 protein could be rescued by surrounding non-neuronal wild-type cells.

Recently studies from a number of laboratories have begun to define the ways in which Hsp70 can inhibit the apoptosis signal transduction pathway. Studies performed in cell lines and immune cells have shown that Hsp70 can block apoptosis at both early (Gabai et al., 1998) and

late (Jaattela et al., 1998) stages in the apoptotic cascade. A functional analysis of the role of the different Hsp70 subdomains in brain cells subjected to SOD1-mutant induced injury will begin to elucidate how Hsp70 protein modulates apoptosis in this setting, and could potentially lead to the development of new therapeutic approaches. Since Hsp70 can block both apoptotic and necrotic cell deaths, it is an especially interesting target for mutant-SOD1 induced toxicity and hence its therapy. Identifying those actions of the chaperones that are most important for blocking injury may possibly lead to the development of novel approaches to lessen damage from both chronic and acute neurodegeneration. One likely direction is the reduction of protein aggregation. Structure-function studies of chaperones will identify which activities and which protein-protein interactions are most significant to different injuries as well as the most broadly protective.

Recently, Kieran et al (2004) provided evidence of a new category of molecules that may ameliorate the disease. These investigators showed that daily treatments with arimoclomol, a co-inducer of heat shock proteins, meaning that it induces expression of more than one Hsp, slows the progress of motor neuron death and consequently extends survival in a transgenic SOD1-G93A mouse model of ALS. The authors documented that arimoclomol induces the phosphorylation of a heat shock protein-inducing factor (Hsf-1), and as a result upregulates expression of Hsp-70 and Hsp-90 (Hargitai et al., 2003). It is thought that the molecule may also upregulate co-chaperones such as Hsp-40, CHIP and Bag.

Several aspects of these new findings are notable. First, arimoclomol prolongs survival when administered after onset of disease symptoms. To date, over 70 drugs have been tested in these mice, however there have been no published reports of compounds that are beneficial when started following disease onset. Furthermore, only a small number of compounds prolonged survival by more than 10%, even when started presymptomatically.

It is highly unlikely that ALS is triggered by a single abnormality or insult to the motor neuron. Rather, the mechanisms that trigger motor neuron degeneration are complex and multiple pathways are implicated such as abnormal protein aggregation, oxidative injury, mitochondrial defects, abnormalities of calcium homeostasis, axonal transport

defects, proteosome dysfunction, immune marker induction, caspase activation, and multiple triggers of apoptotic signalling (Kawamata et al., 1992; Julien 2001; Shaw et al., 2003). The implication of multiple parallel pathways in neurodegeneration suggests that an effective alteration of any one pathway implicated in the process, will not be enough to affect the overall course of the disease (Rosenfeld, 2000). In addition, there is an increasing awareness that identified pathogenic processes may affect select subsets of ALS patients in different ways, suggesting the level of primary importance of different processes, may vary on an individual basis (Eisen et al., 1999; Ro et al., 2003; Shaw et al., 2003).

The observation that the degenerative cascade resulting in motor neuron death is probably multi-factorial provides support for simultaneous administration of multiple therapeutic agents in ALS; as interventions at a single point along the cascade are unlikely to improve function, survival, or quality of life to the same extent as multiple agents. Multiple synchronized interventions may have synergistic effects and result in a more significant benefit.

The difference in presentation and subsequent course of the disease between patients suffering from ALS is well known and striking. In some patients selective populations of motor neurons are spared whereas they are markedly affected in others. Although such selectivity can be put down to biological variability, it is also consistent with the hypothesis that phenotype variability may be the result of different pathophysiologic mechanisms. In picking subjects for a clinical trial, it is desirable to obtain a homogeneous population for the study. However, recent clinical trials utilising a single pharmacologic intervention have incorporated subjects with a surprising heterogeneity in the rate of progression, site of onset, and resulting functional limitations (Jackson et al., 2001; Miller et al., 2001; Cudkowicz et al., 2003; Groeneveld et al., 2003).

To date, multiple clinical trials have been completed using individual agents including recombinant human ciliary neurotrophic factor (rHCNTF), riluzole, insulin-like growth factor-1 (IGF-1), topiramate, alpha-tocopherol (vitamin E), creatine, selegine, nimodipine, verapamil, and gabapentin (Bensimon et al., 1994; Borasio et al., 1998; Miller et al., 1996 and2001; Jackson et al., 2001; Kwiecinski et al., 2001; Desnuelle et al., 2001;

Cudkowicz et al., 2003; Groeneveld et al., 2003). Regrettably however, none of these single agents has produced significant benefit. Currently, as mentioned previously, Riluzole is the only FDA-approved drug for ALS patients, and has a modest effect on survival, respiratory capacity and rate of declining strength, but not on total strength, quality of life, or functional capacity (Lacomblez et al., 1996). From data obtained from animal models, it is known that anti-glutamate agents are thought to affect the initiation but not the propagation of motor neuron degeneration; the converse being true for antioxidant agents (Gurney et al., 1996; Torreilles et al., 1999; Ochs et al., 2000; Simpson et al., 2003).

A combinational drug treatment that addresses different pathophysiologic mechanisms makes sense and is necessary to achieve the most significant benefit (Eisen et al., 1999; Mitsumoto et al, 2001; Carter et al., 2003). Several reports, utilising different laboratory models of motor neuron degeneration, support the benefit of combination drug therapy (Iwasaki et al., 1999; Bilak et al., 2001; Mohammadi et al., 2001; Kriz et al., 2003; Nagano et al., 1999 and2003; Zhang et al., 2003). Some success has been obtained from limited combination drug trials, suggesting synergistic effects between different drugs (Stevic et al., 1998; Cudkowicz et al., 2003). In this study the combinational effect of Hsp27 and Hsp70 was more effective in reducing mutant-associated toxicity than either of the single applications respectively.

The results presented in this chapter suggest the following hypothesis to explain how mutations of SOD1 may trigger neurodegeneration. SOD1 is an abundant protein that, when altered structurally as a direct consequence of mutations or subsequent post-translational modification, becomes denatured at an increased rate. As a result of SOD1's abundance, it is likely to re-route a substantial portion of the chaperone systems toward refolding the denatured SOD1 or targeting it for degradation by proteasomes. The depletion of the pool of free chaperones would render cells expressing mutant SOD1 particularly vulnerable to physiological and environmental stresses, and would reduce the efficiency of several cellular processes dependent on chaperone proteins, and additionally might allow formation of aggregates of denatured protein. Since most cells possess the ability to up-regulate the levels of many chaperone proteins, they remain relatively healthy. However, there is evidence that motor neurons may be relatively deficient in their ability to induce certain Hsps with

chaperoning activity upon exposure to stress (Manzerra and Brown, 1992; Morrison-Bogorad et al., 1994; Satoh and Kim, 1995). As a result this may render them particularly vulnerable to damage from mutant SOD-1. In addition, due to the fact that they are also subjected to physiological stresses, including a high-level excitatory input, this may add to the overall burden on the chaperone systems.

If depletion of free chaperones were a significant factor in the toxicity of mutant SOD1, then by increasing the levels of chaperone proteins one would be able to confer protection against it. Durham et al. (1997) used intranuclear microinjection, to express G93A-mutant-SOD1 in primary motor neurons of dissociated cultures of murine spinal cord, which resulted in formation of cytoplasmic aggregates of SOD-1 and loss of viability over a 2-week period. Durham et al. (1997) found that co-injection of expression vector encoding the murine inducible Hsp70 reduced the toxicity of mutant SOD1, dramatically decreased the percentage of motor neurons with SOD1 aggregates, and also prolonged survival.

Using three different experimental models, we have demonstrated that increased expression of Hsps protects cells from the toxicity of SOD1 mutants. Molecular chaperones are essential for several processes, including resistance to stress and proper protein folding, intracellular transport, and degradation. The diversion of the chaperones to protect the cell from toxicity of mutant SOD1 would have grave consequences, particularly if additional demands were exerted through environmental stresses. Motor neurons would be particularly vulnerable to mutant SOD1 associated toxicity for a number of reasons: (1) they express high levels of SOD1 (2) induction of protective chaperone proteins in response to stress may be less robust in motor neurons than compared to other cells and (3) they are subjected to additional physiological stresses including a high level of excitatory synaptic input.

The work presented here provides a new therapeutic approach to this disease-the enhancement of the heat shock response. Indirectly, these findings support the hypothesis that the cellular Achilles heel in hereditary and may be also sporadic ALS is the accumulation of misfolded SOD1 protein and any associated binding proteins. These accumulations could have calamitous downstream consequences, including direct toxicity to subcellular organelles and proteosomal dysfunction. The work presented here is in accordance with

previous *in vitro* studies showing that expression of Hsp70 blunts the neurotoxic effects of acutely expressed mutant SOD1 (Shinder et al., 2001; Bruening et al., 1999; Okado-Matsumoto et al., 2002).

The results presented here also suggest that manipulating the heat shock pathway at more than one place achieves a more profound neuroprotection than altering levels of individual Hsp molecules. In accordance with the findings of arimoclomol other activators of the heat shock response may also prove beneficial in the SOD1-G93A mice and other related diseases for instance carbenoxolone (Nagayama et al., 2001), an anti-ulcer drug that directly activates the Hsp70 promoter; the herbal compound celastrol (Allison et al., 2001); and the anti-inflammatory drug indomethacin (Lee et al., 1995), which, among other mechanisms, activates DNA binding of HSF-1.

It will be important to establish whether the enhancement of heat shock and protein chaperone responses is beneficial in cases of ALS that are not a consequence of SOD1 gene mutations. Due to the existence of considerable overlap between the cellular and bio-chemical phenotypes of sporadic and familial ALS it is anticipated that Hsp induction will be beneficial for all types of ALS. However, in the absence of an animal model for sporadic ALS, the only way to resolve this would be through human clinical trials.

The neuroprotective effects of Hsp activation may also extend to other conditions for instance stroke, or trauma of the brain and spinal cord. An analog of arimoclomol, bimoclomol, induces Hsp70 expression and prevents neuronal degeneration in a neonatal model of motor (Kalmar et al., 2002) and sensory (Kalmar et al., 2003) neuron death, and in models of diabetic neuropathies (Biro et al., 1997), cardiovascular injury (Polakowski et al., 2002), as well as ischemia/reperfusion injury (Lubbers et al., 2002). Hargitai et al., (2003) demonstrated that bimoclomol mediates its co-inducer effect on heat shock protein gene expression and cytoprotective action via HSF-1. Although further studies are required to examine the precise manner in which bimoclomol exerts its effect on HSF-1 or its complexes with heat shock proteins, this appears to involve a moderately enhanced phosphorylation of HSF-1 in addition to prolonged binding of HSF-1 to DNA. Bimoclomol-induced fluidization of cellular membranes (Török et al., 2003) may also lower the threshold

of the heat shock response, and as a result contribute to the chaperone co-inductive property (Vigh et al., 1997) of this drug. It may also be fruitful and important to study further how arimoclomol works. For instance, which kinases and phosphorylases impinge on HSF-1, and whether these molecules may potentially be drug targets. Substances that upregulate Hsps and their co-chaperones may be of therapeutic importance in other neurodegenerative diseases, many of which are a consequence of protein instability (for example, amyloid-β in Alzheimer disease, α-synuclein in Parkinson disease, huntingtin in Huntington disease, and prion protein in Creutzfeld-Jacob disease).

Knowledge of the genetic defects triggering neurodegeneration in ALS has provided insight into molecular pathogenesis, and consequently led to the development of disease models in transgenic mice and simple *in vitro* systems. Ultimately it is hoped these powerful tools will facilitate the discovery of new drugs and therapies. The findings presented here are considered to be an important step in this direction.

Overall, the findings presented in this chapter do not provide sufficient evidence for the full understanding of the mechanism of toxicity of FALS-associated mutant-SOD1 or of the precise mechanism of action via which Hsp27 and/or Hsp70 exert their protective effects. However, they clearly suggest (a) that both Hsp27 and Hsp70 are of potential therapeutic benefit toward FALS-associated mutant SOD1 toxicity and (b) that a combinational approach utilizing dual expression of Hsp27 and Hsp70 together maybe more effective than expression of single Hsps. Further suggestions for future experiments utilising the simple cellular models presented here or more advanced systems to confirm these findings - such as more advanced *in vivo* models - are discussed in the next chapter, along with a further evaluation of the present study.

# CHAPTER 6

# GENERAL DISCUSSION

# 6. Discussion

The work presented in this thesis demonstrates the effectiveness of using a simple neuronal cellular model, in addition to a primary cell model of mutant-SOD1-induced toxicity and an efficient gene delivery method based on HSV-1 vectors, in order to test hypotheses relevant to FALS-related pathogenesis and to the neuroprotective effects of heat shock proteins. This chapter aims to discuss general aspects, as well as the advantages and limitations of the work presented here and in addition suggest further experiments, which may be carried out (more details are included in the individual Discussion sections of the respective Chapters 3, 4 and 5). Therefore, Chapter 3 described the establishment of the *in vitro* cellular model and characterisation of its response to a wide range of stresses relevant to FALS. Having demonstrated certain effects in this system, which result from the over-expression of either wt or G93A or G93R-mutant-SOD1 forms, it was then possible to move forward and ask whether or not Hsp over-expression could possibly alleviate or prevent the deleterious effects of mutant-SOD1 under conditions of stress (Chapter 4); and then investigate potential mechanisms of action of Hsp27 and Hsp70 (Chapter 4) as well as the effects of ApoE alleles in relation to FALS; and then further investigate the effects of wt-SOD1 and its FALS-associated mutant forms and Hsps in a primary cell culture model in order to confirm the findings (Chapter 5).

The following sections will highlight how the findings presented in the last three chapters compare with the findings reported in the literature, before proceeding to summarise the overall advantages and the limitations of the models presented here. Lastly, some further experiments are suggested that may be carried out to extend the work presented in this thesis.

The results presented in Chapter 3 describe the establishment and characterisation of the *in vitro* model system where wt-SOD1 and the disease-associated SOD1-mutant forms are over-expressed. It was shown that the response of the cell lines to various death-inducing stimuli depends on both the type of stress and the form of SOD1, which is over-expressed. Wt-SOD1 expression protected the neuronal cells against all stresses tested whereas both the SOD1-mutants were more susceptible to death (also, see Patel et al., 2002 in the appendix).

There is substantial evidence (also discussed in Chapter 3) indicating that increased levels of wt-SOD1 are protective (Greenlund et al., 1995; Rabizadeh et al., 1995; Patel et al., 2002) whilst expression of the mutants is deleterious *in vitro* (Rabizadeh et al., 1995; Patel et al., 2002), and *in vivo* (Gurney et al., 1994; Dal Canto and Gurney 1995 & 1997). Evidence of apoptosis is observed in transgenic mice overexpressing FALS associated SOD1 mutations from the elevated levels of caspase-1 and caspase-3 (Li et al., 2000; Pasinelli et al., 2000) and from the observation that neuroprotection can be achieved by crossing these SOD1 transgenics with mice overexpressing Bcl-2 (Kostic et al., 1997). Cell culture experiments further substantiate the pro-apoptotic properties of FALS associated SOD1 mutations (Rabizadeh et al., 1995; Patel et al., 2002).

The protective effect of the wild-type SOD1 against neuronal apoptosis has been demonstrated in several studies (Greenlund et al., 1995; Rabizadeh et al., 1995) and this has also been shown in our studies. Wild type SOD1 protein provided protection against a wide range of stresses including serum removal both in the absence and presence of 1μM retinoic acid, staurosporine administration, IFN-γ treatment, camptothecin addition, glutamate administration, hydrogen peroxide treatment and ischemia/reperfusion. Hence, in a wide range of different situations the wild-type SOD1 is protective however the disease-associated SOD1 mutants G93A and G93R exhibited an enhanced level of cell death on exposure to the same stresses. The results of the present study extends the work of Rabizadeh et *al.,* (1995) who demonstrated that the SOD1 mutations G37R and A4V associated with ALS converted the anti-apoptotic SOD1 gene into a pro-apoptotic gene. Furthermore, in the present study the G93R mutation was observed to be a lot more severe compared to the G93A mutation paralleling the much more severe disease phenotype and early onset observed in ALS patients with this mutation.

As a summary, comparison of this model to mice and *in vitro* FALS models (reviewed in Chapter 1 and discussed in Chapter 3) suggest that all the findings in this model are in agreement the findings of others, on both the protective effect of wt-SOD1 under all stresses tested and the role of mutant-SOD1 in enhancing toxicity (Patel et al., 2002). This work incorporates a wide range of FALS relevant stresses, in addition to some stresses, which have previously not been investigated.

Chapter 4 presented experimental data that provided a novel neuroprotective role for Hsp27 and Hsp70 both singly and to a greater extent in combination, against FALS-associated SOD1 mutant forms expressed in neuronal cells (see also in the appendix Patel et al., 2005). Hsp27, Hsp70 and the Hsp27 and Hsp70 combination have a potent protective effect against mutant SOD1-induced toxicity under all the pro-apoptotic and oxidative stresses tested. This is a novel finding in the context of FALS models, and agrees with numerous studies in the literature that support the protective function of Hsp27 and Hsp70 against many stresses resulting in apoptosis (see Chapter 5 discussion below). In addition, investigation into the effects of the ApoE alleles revealed the protective effects of ApoE2 over ApoE4 in the SOD1 mutant expressing cells. ApoE4 addition appeared to increase cell death across all cells and appeared to be deleterious for the cells.

Although studies have examined the protective effects of over-expression of Hsps, none have investigated conclusively the protective effects of Hsp27 or Hsp70 over-expression, or combined effects of both together, with respect to FALS-associated SOD1 mutants. Notably, Hsps are found to be present in aggresomes in cell culture models (Durham et al., 1997) and in FALS patients together with mutant SOD1 and other Hsps. Recently, Shimura et al. (2004) reported Hsp27 rescues pathological hyperphosphorylated tau-mediated cell death in a human cortical neuronal cell line. Interestingly, in human AD brain, Hsp27 was shown to bind to pathological hyperphosphorylated tau but not non-phosphorylated tau. The formation of this complex *in vitro* results in a decrease in its concentration by assisting its degradation and dephosphorylation.

In the present study Hsp27, Hsp70 and the Hsp27 and Hsp70 combination all conferred protection to the G93A and G93R SOD1 mutants against a wide range of lethal stimuli. Hsp27 was more effective in reducing death in the G93A mutant than Hsp70 whereas in the G93R mutant the converse was true. However the combination of Hsp27 and Hsp70 was the most effective in reducing death in the SOD1 mutants. Interestingly, wt-SOD1 cells exhibited increased levels of death on infection with the Hsp's, with Hsp70 bringing about a larger increase in death than Hsp27. In addition the enhanced cell death observed in the wt-SOD1 cells on infection with Hsp27 or Hsp70 alone was abolished on

expression of the combination of Hsp27 and Hsp70 suggesting that Hsp27 and Hsp70 may act in synergy.

A significant study by Bruening *et al.* (1999), in a mammalian system, reported that increasing the levels of Hsp70 reduced formation of mutant SOD-containing proteinaceous aggregates and prolonged survival. As yet it remains controversial as to whether cytoplasmic mutant SOD1 aggregates are toxic (Bruijn et al., 1998) or not (Cummings et al., 1999; Johnston et al., 2000; Kopito. 2000). Previous studies in neuronal cells and cultured primary motor neurons have demonstrated that the inhibition of cytoplasmic aggregate formation by induction of heat shock protein Hsp70 assured cell survival at an early stage but did not prevent eventual cell death at the late stage in the *in vitro* models of FALS (Bruening et al., 1999; Takeuchi et al., 2002).

Work conducted by Zourlidou et al. (2004) on the protective effects of Hsps in a neuronal cell model of α-synuclein toxicity found Hsp70 did not provide a strong protective effect against a range of stresses, whilst simultaneous overexpression of Hsp27 and Hsp70 was as protective as Hsp27 expression alone. Recently, Klucken et al. (2004) reported the first evidence for an effect of Hsp70 on reducing high molecular weight and detergent insoluble α-Syn species in an *in vivo* mammalian model. The study also reported protection from α-Syn toxicity by Hsp70 in an *in vitro* model of α-Syn aggregation and reduction of detergent insoluble α-Syn species. Over-expression of Hsp70 in several *in vitro* and *in vivo* models of polyQ disease has been demonstrated to be efficient in suppressing polyQ aggregation (Muchowski et al., 2000) or both aggregation and toxicity (Sherman and Goldberg, 2001). In a mouse model of SBMA, over-expression of human inducible Hsp70 led to a reduction in the levels of aggregated and monomeric mutant androgen receptor (AR) in neuronal nuclei and alleviated disease phenotype (Adachi et al., 2003). In addition, in a SCA1 model over-expression of Hsp70 did not avert the formation of nuclear inclusions in Purkinje cells but suppressed degeneration and improved motor performance (Cummings et al., 2001). Phenotype rescue, as measured by survival, rotarod analysis and gait analysis, was seen on homozygous over-expression of Hsp70 with Hsp70 reducing the levels of both aggregated and soluble AR detected in the nucleus. Hsp70 over-expression may aid in degradation of the mutant AR protein via the ubiquitin-proteasome system.

In the model presented here, the main mode of cell death induced by the various stimuli appears to be apoptosis. Hence the findings in this thesis could be explained partly by the fact that both Hsp27 and Hsp70 have multiple anti-apoptotic actions (Garrido et al., 2001; Concannon et al., 2003; Parcellier et al., 2003). The precise mechanism of protection of Hsp27 and Hsp70 in various situations is obviously very complex and as yet remains incompletely understood. However we believe that the Hsps provide more than a general cytoprotective effect and impose their protective effect on the SOD1 mutant probably through restoration of normal protein conformation rather than degradation of misfolded protein, as addition of Hsps lowers levels of death in both the mutants by a greater amount than is seen in control cells without SOD overexpression. The reduction in death of the mutants, beyond the levels of control cells would be expected if the mutant was refolded to obtain wild-type conformation and thereby able to exert its wild-type anti-apoptotic properties through over-expression of wt-SOD1 protein.

In the case of wt-SOD1, Hsp27 or Hsp70 expression resulted in an increase in cell death. This may be the result of increased levels of oxidative stress through increased levels of basal SOD1, known to be harmful to the cell (Omar et al., 1990) (discussed in Chapter 4). The Hsp27 and Hsp70 combination in the wt-SOD1 cells brought levels of cell death down towards control levels. This maybe brought about by the combination of Hsp27 and Hsp70 activating a proteolytic pathway and degrading any excess wt-SOD1 or the combination self-regulating each other and preventing over-excessive production of wt-SOD1. Alternatively, the two factors together may have a subsequently potent protective effect to neutralise the damage brought about by a large excess of wt-SOD1. Therefore depending on the conditions the Hsp27 and Hsp70 combination may protect against apoptotic cell death by interfering with the caspase cascade (discussed in Chapter 4), or assist in the refolding of mutant protein or assist in the degradation of the protein via the proteasome under the conditions described and at the chosen time points.

The work presented in Chapter 5 provides further support to the findings of the previous two chapters, through work in DRGs from rats, mice and transgenic mice to confirm that Hsp27, Hsp70 and to a greater extent the Hsp27 and Hsp70 combination provide protection against mutant SOD1 induced toxicity and that the mechanism of

cytoprotection of Hsp27 and/or Hsp70 may be through interference with caspase activation in cells in which mutant SOD1 is expressed. Nevertheless, and although speculative, it is more likely that Hsp27 or Hsp70 confer protection through involvement at a number of sites/pathways and this should be further investigated.

The results support the idea that it is the anti-apoptotic activity of Hsp27 or Hsp70, which is responsible for protection against cell death. The involvement of apoptosis in FALS and also the role of Hsps in suppressing apoptotic pathways were discussed extensively in Chapter 1 along with the role of mitochondria in both FALS and apoptosis. In summary, there exists work conducted by others that strongly supports the occurrence of apoptosis in FALS models and in FALS brain (Vila and Przedborski, 2003), as well as work supporting the capabilities of Hsp27 or Hsp70 in suppressing it (Concannon et al., 2003; Parcellier et al., 2003a; Beere, 2004).

To further investigate the protective effects of over-expression of Hsp27, Hsp70 and the Hsp27 and Hsp70 combination against mutant SOD1 induced toxicity, quantification of the cell death in primary neurons was performed for three distinct stresses all relevant to FALS (NGF withdrawal, IFN-γ and staurosporine). Both Hsp27 and Hsp70 and to a greater extent the Hsp27 and Hsp70 combination, conferred protection against mutant SOD1 induced toxicity. Although the findings presented in Chapter 5 do not provide a full understanding of the mechanism of protection in the model of mutant-SOD1 induced toxicity, they provide significant evidence on the interference of Hsp27, Hsp70 and the Hsp27 and Hsp70 combination with caspases and the apoptotic cascade events. It is possible that multiple sites of action as well as multiple activities of both Hsp27 and Hsp70 are involved in this protection. For instance, apart from suppressing apoptosis (Beere et al., 2004), Hsp27 may act by (a) stabilising actin microfilaments (Lavoie et al., 1995) and interacting with cytoskeleton components to stabilise the cytoskeleton (see review of Hsp27 roles in Chapter 1), (b) by increasing the anti-oxidant defence of cells by decreasing reactive oxygen species cell content (Mehlen et al., 1996), (c) assisting in proteasomal degradation (Parcellier et al., 2003). On the other hand, Hsp70 apart from its ability to suppress apoptosis is also thought to protect cells from energy deprivation and/or ATP depletion associated

with cell death (Wong et al., 1998). Furthermore, Hsp70 has also been shown to avert JNK activation (Meriin et al., 1999; Park et al., 2001).

The mechanism by which Hsp27 exerts its protective effects may be through its ability to maintain redox homeostasis and mitochondrial stability. The suggestion that Hsps exert their protective roles at the level of the mitochondrion is not an entirely new concept. Previously Polla *et al.* (1996) proposed that mitochondria are the targets of the protective effects of Hsps against oxidative stress. Mutant SOD1 has been shown to localise to the mitochondria where it sets off caspase-dependent cell death (Takeuchi et al., 2002). It is possible that mutant SOD1 exerts its effects on mitochondrial SOD2 by relocating to the mitochondria where the SOD1 mutant catalyses the reaction of superoxide radical ($O_2^-$) with nitric oxide (NO) to generate peroxynitrite ($ONOO^-$). Hsp27 can increase intracellular levels of glutathione (Mehlen et al., 1996) and this may be a possible additional mechanism by which Hsp27 exerts its protective effect. The role of the mitochondrion in FALS is becoming increasingly important. Therefore, the protective effects of Hsp27 presented here may act by protecting mitochondrial integrity, however further work need to be done to confirm this.

*In vitro* cellular models have been used extensively in neurodegenerative disorders and have provided a substantial amount of valuable knowledge. Some of these findings were extended through *in vivo* experiments or confirmed through studies on human post mortem material. In the same way, the methodology used here is not novel however the model systems presented here have contributed overall to the field; this will be explained next alongside some of its limitations.

The limitations that accompany the *in vitro* model systems used in this study are mainly due to the fact that they are *in vitro* systems utilising a non-human hybrid cell line or rat or mouse DRGs. However, such limitations do not necessarily rule out the significance of these findings, as in total three model systems have been utilised in this study. However further work needs to be carried out in *in-vivo* systems. In addition it should be noted that the majority of ALS cases are not linked to SOD1 mutations.

Neurodegenerative diseases like ALS are often very complex and not completely understood at the molecular pathological level. There are additional difficulties imposed by the fact that they are age related disorders and progressive diseases where usually more than one parameter accounts for their pathology, with environmental triggers and genetic predisposition. Therefore, the extent to which a simple cellular model can provide a better understanding of the disease pathogenesis is relatively limited. However, by reviewing the biology of Hsps and of SOD1, the cellular model system presented here was able to demonstrate a novel neuroprotective role for Hsp27, Hsp70 and the Hsp27 and Hsp70 combination on mutant-SOD1 induced toxicity. The role of Hsp27 and Hsp70 in conferring protection on this system is not surprising, in view of evidence of the anti-apoptotic functions for both Hsp27 and Hsp70 and protection from ROS, ATP depletion and a range of stimuli in non-neuronal and neuronal cells or *in vivo* systems (see review in Chapter 1).

As discussed in the previous chapters, advantages of the system presented here include the fact that ND7 cells are well characterised neuronal mammalian cells, and this present system has been well characterised (Chapter 3). The viral vectors used in the present studies provided a highly efficient mechanism of Hsp gene delivery to the neuronal cells and the DRGs, with a transduction efficiency of between 90-100%, as discussed in Chapter 4 & 5. Strong evidence on the protective effect of Hsp27, Hsp70 and the Hsp27 and Hsp70 combination against various apoptosis-inducing stresses was provided by two different methods of quantitative cell death assessment (trypan blue exclusion assay and TUNEL), as shown in Chapter 4. These are both valid and widely used methods for the assessment of cell death with TUNEL being more specifically used for the assessment of the apoptotic index.

As a result of time limitations there was not sufficient time to study the role of the proteosome, which has only recently been studied in other *in vitro* systems. It would be interesting to perform experiments involving proteasome activity assays and see if Hsp27, Hsp70 or the combination of both rescues the cells from a potentially toxic proteasomal impairment.

In order to extend the study of the Hsp27, Hsp70-mediated mechanisms that confer protection in our system, it would be interesting to see if there are enhanced levels of ROS

as a result of mutant SOD1 expression and if so, whether Hsp27/Hsp70 or both suppress their formation and hence susceptibility to death in our system. In addition, some other stresses could be investigated, such as those inducing oxidative damage, proteasome inhibitors, inhibitors of the mitochondrial complex and so on, as these are relevant agents to the disease pathology and have been extensively used by others. Finally, it could be of interest to investigate the mitochondrial activity, for instance, by using commercially available assays, such as caspase activity assays, and then proceed to see if cytochrome c release is blocked by Hsp27, Hsp70 or the combination.

Aggregate formation by SOD1 and its mutant forms was not investigated here and hence could be further investigated, as this could explain the increased cell death observed on treatment of wt-SOD1 cells with the Hsp27 and Hsp70 virus, as well as the protective effects observed in the mutants. Therefore, immunocytochemical or electron microscopy studies may shed some light on this aspect of the cellular model. Alternatively, through anti-sense, dominant negative constructs or RNA interference technology, one may ask whether compromising the inherent Hsp machinery in these cells sensitises them by a greater extent to mutant SOD1 over-expression in the presence or absence of stress.

Furthermore, along with the use of viruses to efficiently over-express individual or combined Hsps in cells, drugs could have also been used in order to (a) initially characterise their effectiveness in Hsp induction through the drug-induced stress response and (b) subsequently assess cytoprotection. As chaperones are multifunctional proteins it is possible that their depletion may impair mechanisms, which are independent of protein folding. Evidence suggests that the abundance and relative levels of chaperones may be critical in determining cellular signalling (Nollen and Morimoto, 2002; Young et al., 2003).

The pharmacological or other Hsp inducing strategies should aim to (a) replace potential "losses" of chaperones, for instance those that result through sequestration in aggregates, which results in depletion of the cellular pool of Hsps needed for other functions in the cell and (b) induce Hsps at higher levels than normal to assist the cell to cope with stresses that would otherwise result in malfunction or death. Pharmacological modulation of heat shock protein levels may represent a potential therapeutic strategy toward disorders like

FALS with prominent protein aggregation. A number of compounds have been reported to initiate a heat shock response in a wide range of cell lines.

Finally, confirmation of the findings presented in this study could be obtained through *in vivo* investigations by crossing mice over-expressing mutant SOD1 with available Hsp27, Hsp70 or Hsp27 and Hsp70 transgenic mice, to assess whether the phenotype is altered and if so, conduct biochemical investigations to determine how this is achieved.

Whatever the future holds, the work presented in this thesis is of significance in the field of FALS and heat shock protein biology. This is due to the fact that as yet, no effective treatment exists for FALS and heat shock proteins represent therapeutic targets and potent therapeutic agents for the modulation of neurotoxicity in neurodegenerative disorders. Before any definitive conclusions can be drawn this work needs to be extended and validated in more complex systems *in vivo*. Better understanding of FALS and its molecular pathogenesis will assist in the development of novel therapeutic interventions, which may involve manipulation of the heat shock response pharmacologically or by means of gene therapy.

Although the cellular model presented here is not the ideal cellular model to study ALS - it remains a very useful mammalian neuronal system where an interesting effect of wt-SOD1 and the FALS-associated mutants has been observed (Chapter 3). This system has an obvious advantage over the reported use of various non-neuronal or non-mammalian systems. It has allowed the testing for the first time of the protective effect of various Hsps both singly and in combination, against the mutant SOD1-induced cell death in a mammalian neuronal system. Most importantly, this system helped to conclude that Hsp27, Hsp70 and to a greater extent the Hsp27 and Hsp70 combination is a potent neuroprotective agent against mutant SOD1-associated toxicity; this is the first report to show alleviation of mutant SOD1-neurotoxicity in an *in vitro* mammalian neuronal system by Hsp27 and by the combination of Hsp27 and Hsp70 (Patel et al., 2005).

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